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From the Pharmacological Institute, University of Upsala, Sweden, and the Ophthalmological Research Unit, Medical Research Council, London.

On the Origin of the Ascorbic Acid in the Aqueous Humour of Guinea-Pigs and Rabbits.

By

ERNST BÁRÁNY and MAURICE E. LANGHAM.

Received 14 January 1955.

The unsatisfactory state of our knowledge concerning the origin of the high concentration of ascorbic acid in the aqueous humour of many species has been pointed out by BELLOWS (1944) and LINNÉR (1952) in reviews of the relevant literature. In rabbits, guinea-pigs and human beings it has been shown that a rise in the concentration of ascorbic acid in the plasma leads to an increase in the concentration in the aqueous humour to a ceiling level, even though the raised plasma level may be considerably below that of the aqueous humour (KINSEY 1947, LANGHAM 1950, LINNÉR 1952, 1954). Recently LINNÉR (1952) has suggested that the amount of ascorbic acid secreted into the aqueous humour in these species may be dependent on the quantity reaching the site of transfer in unit time, and has used this compound to estimate changes in the rate of flow of plasma through the ciliary processes in a manner analogous to the use of p-amino-hippuric acid in studies of renal function.

To determine the absolute rate of flow of plasma by Linnér's method it is necessary to know if ascorbic acid is synthesised within the globe. Furthermore, one has to know the nature of the substance in the plasma that later appears as ascorbic acid

in the aqueous humour. In this respect the rôle of the dehydro-ascorbic acid of the plasma is of interest for it has been suggested by MÜLLER and BUSCHKE (1934) that this substance is the immediate source in the plasma of the ascorbic acid in the aqueous humour.

In the present study a more specific method for the determination of ascorbic acid in the plasma than used by previous investigators in the field has been applied. It has been used to investigate the relation between the concentrations of ascorbic acid in the plasma and aqueous humour of the guinea-pig and rabbit and the possible synthesis of ascorbic acid in the eye. Further, an attempt has been made to assess the relative importance of ascorbic and dehydro-ascorbic acids as precursors in the mechanism of transfer.

Methods.

Experimental.

Guinea-pigs of each sex, weighing 300—700 g, were used. They were kept on a normal diet of hay, crushed oats, turnips and water *ad lib.* In some cases freshly cut grass was added. In other experiments a scorbutogenic diet was used: 4.5 parts wheat bran, 2.5 parts crushed oats and 13.0 parts of dried whole milk. This mixture was heated for 2 hours at 110°C in shallow pans with free access of air. The diet produces virtual depletion of the ascorbic acid stores in one week. The rabbits used were adults of each sex, weighing 1.9—2.6 kg and kept on *Diet 18* pellets (Associated London Flour Millers), water *ad lib.* and hay once a week. All experiments were made on conscious animals, blood samples being obtained by heart puncture with coarse needles mounted on all-glass syringes moistened with a solution of heparin in normal saline. The blood was run into chilled centrifuge tubes and spun for 7 to 15 minutes. Aqueous humour was removed following paracentesis of the anterior chamber under local anaesthesia with lidocaine or pantocaine.

The maximal accumulation of ascorbic acid in the aqueous humour was studied using an injection technique. Conscious guinea-pigs were subjected to a continuous subcutaneous infusion of a partially neutralised (pH 5.5) isotonic solution of ascorbic acid for 6 hours, the plasma level being kept well above that necessary to saturate the transfer mechanism but below the concentration in the aqueous humour. A period of six hours is sufficient to allow a steady state to be reached in the aqueous humour to within a few per cent. The technique was varied for rabbits in that injections were made subcutaneously at intervals of 30 minutes for a period of 6 hours. This procedure also resulted in a steady state in the aqueous humour provided the fluctuating concentration of ascorbic acid in the plasma remained above the minimum

necessary for saturation and never rose above that in the aqueous humour.

Analytical. The volume of the aqueous humour in the guinea-pig was approximately 40 μl and samples were transferred directly into weighed tubes containing 200 μl of a 5 per cent (w/v) solution of metaphosphoric acid. The quantity of aqueous humour was then determined by weight difference and the ascorbic acid estimated with 2,6 dichlorophenol-indophenol, the titration being completed within 30 sec. The volume of the aqueous humour of the rabbit is approximately 250 μl. 200 μl were pipetted into 200 μl of a solution containing 8 per cent trichloroacetic acid (w/v) and 1.5 per cent metaphosphoric acid (w/v) and centrifuged. The ascorbic acid in the supernatant solution was then titrated with 2,6 dichlorophenol-indophenol in a citrate phosphate buffer adjusted to pH 4.0. Control experiments showed that the presence of up to 25 mg/100 ml of dehydroascorbic acid did not interfere with the method.

Analysis of the concentrations of ascorbic (AA), dehydro-ascorbic (DHA) and diketogulonic (DKG) acids in the plasma was made by the modification of the osazone technique of Roe, Mills, Oesterling and Damron (1948) described by Heald and Langham (1955). The high specificity of the osazone method has been demonstrated by Roe and Kuether (1943).

Estimations of ascorbic acid in the aqueous humour of the rabbit by the osazone and titrimetric procedures agreed within 5 per cent, but marked differences were found in the aqueous humour of scorbutic guinea-pigs. In 23 eyes of 18 animals which had keen kept on the scorbutogenic diet for 7 days an apparent concentration of 4.1 ± 0.3 mg/100 ml (Arithmetic mean $\pm$ standard error) was found by the titration method whilst in other animals treated identically the osazone method showed a complete absence of Vitamin C. The sensitivity of the osazone method was such that concentrations of 1 mg/100 ml would readily have been detected. In similar experiments on the plasma of guinea-pigs nearly or completely scorbutic, the titration procedure was found consistently to show 0.5—1 mg/100 ml more ascorbic acid present in the plasma than indicated by the osazone reaction.

One limitation of the osazone method was found in studies on scorbutic guinea-pigs receiving high doses of dehydro-ascorbic acid. Experiments with AA added to each sample of plasma analysed showed a decrease in its recovery with increase in concentration of DHA and its degradation products in the sample. On the other hand, AA added to rabbit plasma was fully recovered. A further difficulty is that the values obtained for DHA, owing to its lability, tend to be low.

With a concentration of 1 mg/100 ml of AA in the plasma an extinction of 0.10 was obtained at 540 mμ in a cuvette with a 3 cm light path. Readings were made on a Unicam S.P. 501 spectrophotometer or a Zeiss-Opton Elko-II.

The dehydro-ascorbic acid used was a pure preparation containing 1 mole of methyl alcohol of crystallisation (dehydro-l-ascorbic acid monomethanolate). It was kept at 4°C in a desiccator over silica gel.

Results.

The plasma-aqueous humour relation in guinea-pigs and rabbits. To obtain the full range of concentrations of ascorbic acid in the aqueous humour and plasma some guinea pigs were put on a normal diet, some on a scorbutogenic diet and some were given

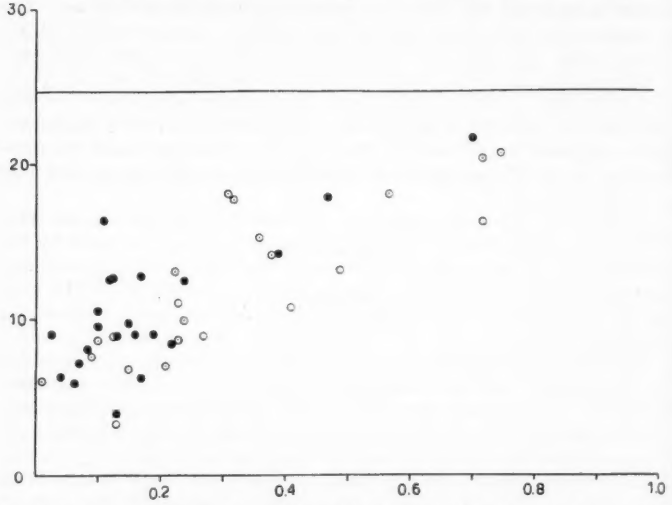

Fig. 1. The concentration of ascorbic acid in the plasma and aqueous humour of the guinea-pig in conditions of steady state.

Abscissa: plasma concentration, ordinate: aqueous concentration, both in mg/100 ml. Each point represents the mean of the two eyes. Filled in circles are from untreated animals while the open circles are from animals given AA. The horizontal line is the mean concentration of ascorbic acid in the aqueous humour when the transfer mechanism is saturated (see text).

ascorbic acid subcutaneously or orally at least 16 hours preceding the experiment.

The relation of the concentration of ascorbic acid in the aqueous humour of the guinea-pig to that in the plasma is recorded in Fig. 1. There appears to be no difference between results obtained with animals previously made scorbutic and then given AA, and those obtained with animals on a normal diet. If the 40 points corresponding to concentrations of ascorbic acid in the plasma between zero and 0.5 mg AA/100 ml are treated as a group and a straight line fitted by the method of least squares the following equation is obtained:

$$C_{aq} = 5.6 + 25.8 \times C_{pl}$$

where C_{aq} and C_{pl} represent the concentration in mg AA/100 ml. The equation indicates the presence of some ascorbic acid in the aqueous humour even when its concentration in the plasma is zero. This may be explained by the lack of specificity of the titrimetric method, already mentioned under 'Methods'. A further possible factor contributing to a positive intercept on the ordinate is a curvature towards the abscissa.

In a group of 9 scorbutic guinea-pigs the concentration of AA in the plasma was raised to about 9 mg/100 ml for six hours. At the end of this period the concentration of AA in the aqueous humour was 24.6 ± 1.3 mg/100 ml. This is slightly less than the figure found by LINNÉR (1952) in the control eyes of guinea-pigs with one carotid ligated or one cervical sympathetic cut, but the difference is not significant. The straight line discussed in the preceding paragraph reaches the plateau value of 24.6 mg/100 ml in the aqueous humour at a value close to 0.8 mg/100 ml in the plasma. Because of the gradual change from the rising to the flat part of the curve relating C_{aq} and C_{pl}, saturation of the transport mechanism will need somewhat more than 0.8 mg/100 ml in the plasma.

The presence of dehydro-ascorbic acid in the plasma of normal guinea-pigs was demonstrated in a separate series of experiments. In 23 animals with a mean concentration of 11.4 ± 1.2 mg AA/100 ml of aqueous humour, 0.09 ± 0.01 mg of DHA/100 ml of plasma was found.

The concentration of AA in the aqueous humour of the rabbit and those of AA, DHA and DKG in the plasma are shown in Table 1. There is a low correlation between plasma DHA and AA, r being + 0.4. The regression line is $C_{DHA} = 0.11 + 0.14\ C_{AA}$ where the concentrations are in mg/100 ml. The relation between the concentrations of ascorbic acid in the aqueous humour and in the plasma is recorded in Fig. 2. At the lower end of the range of plasma concentrations, the concentration of AA in the aqueous humour dropped to approximately 14 mg/100 ml, whilst, with an increase in concentration of AA in the plasma to approximately 2 mg/100 ml the concentration of AA in the aqueous humour rose to the "saturation" level. In separate experiments the value of this upper limit was determined by raising the concentration of AA in the plasma to between 5 and 10 mg/100 ml for six hours, a final concentration of 67.5 ± 2.1 mg/100 ml of AA in the aque-

Table 1.

The concentration of ascorbic acid in the aqueous humour together with the corresponding concentration of ascorbic, dehydro-ascorbic and diketo-gulonic acids found in the plasma obtained by cardiac puncture in normal rabbits. Concentrations are expressed as mg/100 ml.

Body weight kg	Pl_{AA}	Pl_{DHA}	Pl_{DKG}	Mean Aq.
2.7	0.18	0.16	0.08	29.6
2.1	0.40	0.17	0.09	37.9
2.3	0.26	0.15	0.08	21.4
2.4	0.48	0.19	0.11	49.1
2.2	0.48	0.25	0.22	44.7
2.1	0.36	0.17	0.22	27.5
2.5	0.36	0.16	0.10	29.2
2.5	0.50	0.13	0.13	30.7
2.3	0.50	0.16	0.13	30.1
1.8	1.32	0.38	0.17	49.6
2.3	0.62	0.27	0.11	33.7
2.0	0.29	0.16	0.07	21.8
2.4	0.33	0.34	0.09	27.2
2.4	0.39	0.18	0.26	35.6
2.3	0.99	0.30	0.24	43.7
2.3	0.28	0.20	0.15	25.9
2.5	0.06	0.01	0.14	15.2
2.4	0.10	0.09	0.22	18.8
2.2	0.50	0.18	0.21	34.0
3.2	0.77	0.44	0.27	35.9
2.0	0.09	0.08	0.08	13.9
2.3	0.08	0.07	0.03	16.9
2.5	0.08	0.10	0.04	19.4
2.1	0.62	0.14	0.25	34.6
2.1	0.89	0.16	0.23	36.4
2.2	0.86	0.07	0.14	43.9

ous humour being recorded in a series of 30 rabbits. The data are not sufficient to decide within narrow limits where saturation occurs but from Fig. 2 it seems that plasma values above 3.0 mg/100 ml are likely to saturate the transfer mechanism. There is no way of depleting the plasma of the living rabbit of AA and it has not been possible to determine whether there is a residue of AA in the aqueous humour at zero plasma level. Consequently the possibility that some of the AA found in the aqueous humour may be synthesised locally within the globe cannot be excluded.

Animals injected with dehydro-ascorbic acid. The results reported in the previous section indicate the concentration of AA in the aqueous humour for various concentrations of AA in the plasma. In these experiments no effort was made to upset the ratio of the concentration of DHA to AA in the blood. These results will now be used as controls in an attempt to answer the question whether,

in animals where the DHA/AA ratio of the plasma is increased, there is under conditions of steady state more AA in the aqueous than would be expected from the level of plasma AA. Such would be the case if the eye could take up DHA from the plasma and convert it into AA.

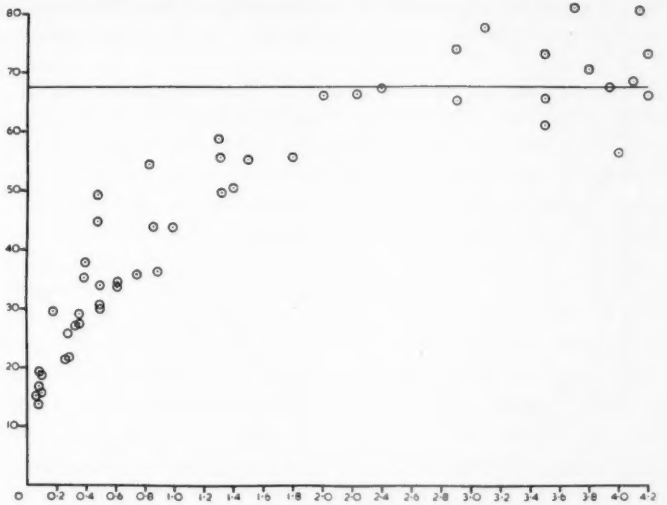

Fig. 2. The concentration of ascorbic acid in the plasma and aqueous humour of the rabbit in conditions of steady state.

Abscissa: plasma concentration, ordinate: aqueous concentration, both in mg/100 ml. Each point represents the mean of the two eyes. The horizontal line is the mean concentration of ascorbic acid in the aqueous humour when the transfer mechanism is saturated (see text).

Plasma AA after repeated injections of DHA. To cause a substantial increase in the DHA/AA ratio of the plasma, scorbutic guinea-pigs were given DHA subcutaneously. Because of the lability of DHA in aqueous solution and the observation in initial experiments that a subcutaneous infusion of a solution of DHA led to no appreciable change in the concentration of AA in the plasma it was decided to inject freshly prepared solutions of DHA at hourly intervals. Fig. 3 shows the plasma concentrations of AA resulting from the subcutaneous injection of 50 mg DHA/kg body weight followed at hourly intervals by further injections of 25 mg/kg. The twenty points comprising the curve were obtained from 41 animals, each of which yielded enough plasma for

only one sample. The points of the curve are therefore means derived from two, or at most three animals. In spite of the extra variation caused by differences between the animals the curve shows that the injection of single doses of DHA causes but transient changes in the plasma AA and that very large amounts of DHA have to be injected to cause a sustained rise. The trend of

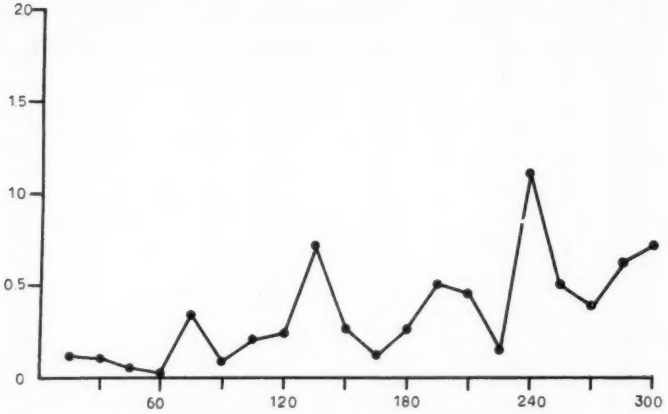

Fig. 3. Average concentration of ascorbic acid in the plasma of scorbutic guinea-pigs given dehydro-ascorbic acid subcutaneously once hourly.

Abscissa: time in minutes after injection of the first dose, ordinate: plasma concentration in mg/100 ml. The first dose was 50 mg/kg body weight, subsequent doses were 25 mg/kg. Data from experiments on 41 animals.

the curve also suggests that even an infusion at constant rate would not have given rise to a flat plasma curve but to one with a steadily increasing slope. The failure of the curve to level off occurred more dramatically in a strictly corresponding series of experiments where twice the amount of DHA was injected; the general shape of the curve was the same but the plasma concentration of AA rose suddenly to 4 mg/100 ml at 210 minutes. The probable explanation of these findings is that the tissues are completely unsaturated at the start of the experiment and take up AA very avidly from the plasma against a concentration gradient (as evident, for instance, in the tables of PENNEY and ZILVA 1946), while towards the end of the experimental period the tissues have become saturated. In other words, the apparent distribution volume of AA decreases with increasing tissue saturation.

In order to obtain steady state conditions it would have been

desirable to arrange an experiment where a constant plasma level of AA was maintained in animals with an increased DHA/AA ratio. This was not found possible however, because of the failure of the concentration of AA in the plasma to attain a constant level. This failure was most marked in guinea-pigs. It has therefore been necessary in this species to derive mathematically from the observed data an 'equivalent steady' plasma concentration of AA.

If the concentration C_{pl} of a substance in the plasma has followed a given course, it will result in a certain concentration in the aqueous humour. The same concentration in the aqueous humour could, however, have been obtained by maintaining a constant concentration of the substance in the plasma for an infinite time. This 'equivalent steady' plasma level E_{pl} is defined by the course of the plasma concentration curve, by the properties of the transfer mechanism and, as a first approximation, by the rate of turnover k_{out} of the substance in the anterior chamber. This is only an approximation since the eye is a multi-chamber system and cannot by described by a single time constant. At the present state of our knowledge it is the only possible approximation for the guinea-pig eye however. In the simplest case, that of proportionality between the steady state concentrations in the plasma and the aqueous humour, the following equation holds:

$$\frac{dE_{pl}}{dt} = k_{out}\,(C_{pl} - E_{pl}) \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (1)$$

In the case of ascorbic acid, the situation is more complex, because proportionality between plasma and aqueous concentration ceases when the transfer mechanism begins to be saturated. It has therefore been necessary to adopt the following procedure in order to derive the 'equivalent steady' plasma level.

In Fig. 4a, the plotted line represents the mean plasma concentration of AA in guinea-pigs given repeated doses of DHA and the isolated point above it, C_e, the plasma concentration of AA in a single animal given DHA according to the same time schedule. The assumption is now made that the curve for the concentration of AA in the plasma of this animal has been of the same shape as the mean curve, only differing in vertical scale by a factor of C_e/C_m, where C_m is the value of the mean plasma curve at that time. Fig. 4b shows the 'assumed' plasma curve leading up to the point found experimentally. The dotted curve is the mean plasma

curve and the dotted horizontal line represents that plasma level at which the transfer mechanism for AA is saturated. Plasma concentrations above this level are considered to have no more influence on the ascorbic acid content of the aqueous humour than those just reaching it. The 'effective' plasma curve therefore is obtained by clipping the highest crests of the 'assumed' curve

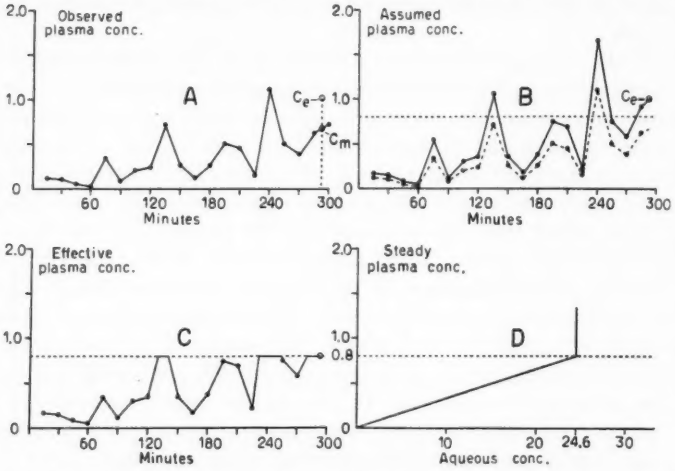

Fig. 4. Experimental and theoretical plasma curves. For interpretation see text.

(Fig. 4c). The C_{pl}-values from this 'effective' curve are finally used in the numerical integration of equation (1).

In the treatment of our data it has been assumed that in all guinea-pigs saturation occurs suddenly at 0.8 mg AA/100 ml in the plasma and that below this level there is simple proportionality, in other words, that the relation between aqueous and plasma levels of AA in conditions of steady state has the form shown in Fig. 4d. The value 0.8 mg/100 ml is very probably somewhat too low but has been chosen because the error is unfavourable to our conclusions. The rate of turnover (k_{out}) of ascorbic acid in the anterior chamber of the guinea-pig eye has been taken as 1.4 per cent per minute, which is the average rate of flow of the aqueous humour found by BÁRÁNY (1951) in scorbutic animals on a similar diet. This value is not critical, because the long duration of the injection experiments makes the 'equivalent steady' plasma level very insensitive to k_{out}. Separate con-

trol experiments in rabbits with the p-amino-hippuric acid method showed that injections of DHA did not affect the rate of flow of the aqueous humour significantly. The numerical integration of equation (1) was done by the step-by-step procedure described by BÁRÁNY and WIRTH (1954). This procedure assumes that the known points of the 'effective' plasma curve are joined by straight lines.

Aqueous AA after injections of DHA. Sixty-eight scorbutic guinea-pigs were injected with DHA. In order to obtain a wide range of concentrations of DHA in the plasma, the animals were given fixed doses regardless of body weight. Preliminary experiments with dosages of 1—6 mg/hour resulted in too small a rise in the concentration of the AA in the aqueous humour. Finally, one of two schedules was adopted throughout, the initial dose being either 20 or 40 mg followed by four doses of either 10 or 20 mg at hourly intervals. The blood and aqueous humour samples were removed 20—40 minutes after the last injection.

The observed plasma concentrations were used for constructing 'assumed' plasma curves as described above. Depending on the dosage received by the animal, either the mean plasma curve illustrated in Fig. 3 or one obtained with twice the dose of DHA was used. Because the samples were taken at different times after the last injection, their concentrations were not all compared with the same point of the mean plasma curve. This is an advantage since the comparison of the observed value with the corresponding point of the mean curve fixes the level of the 'assumed' plasma curve. The 'equivalent steady' plasma levels were derived as described above. They were all lower than the observed values.

Fig. 5 shows the results of the guinea-pig experiments. The concentration of AA in the aqueous humour of each animal has been plotted against the 'equivalent steady' plasma level of AA. For comparison, the regression line derived from part of the data of Fig. 1 is also shown. It is seen that the majority of the points fall below this line. This means that the DHA-injected guinea-pigs had less AA in their aqueous humour than one would have expected from the concentration of AA in their plasma.

In the rabbit, which cannot be depleted of AA, the tissues do not take up AA with the same avidity as in the scorbutic guinea-pig and the plasma concentration of AA resulting from repeated injections of DHA rose to a fairly steady value (Fig. 6). It was therefore unnecessary to calculate an 'equivalent steady'

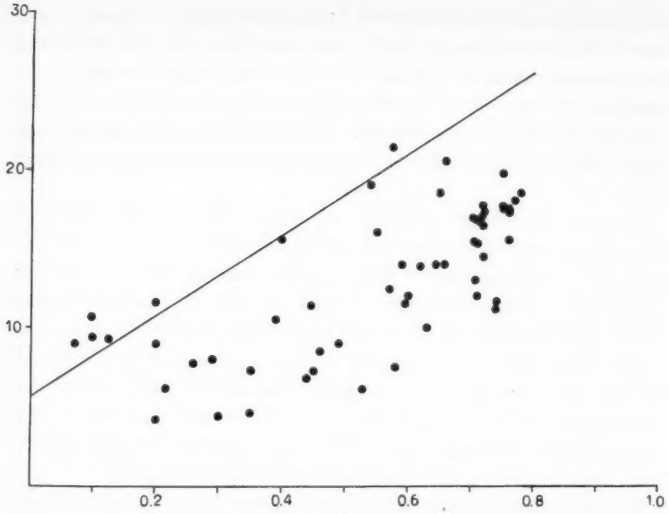

Fig. 5. The concentration of ascorbic acid in the aqueous humour of scorbutic guinea-pigs injected with dehydro-ascorbic acid.

Abscissa: 'equivalent steady' plasma concentration of AA, ordinate: aqueous concentration, both in mg/100 ml. Each point is the mean of the two eyes of the same animal. The line in the figure is that derived for animals not given DHA.

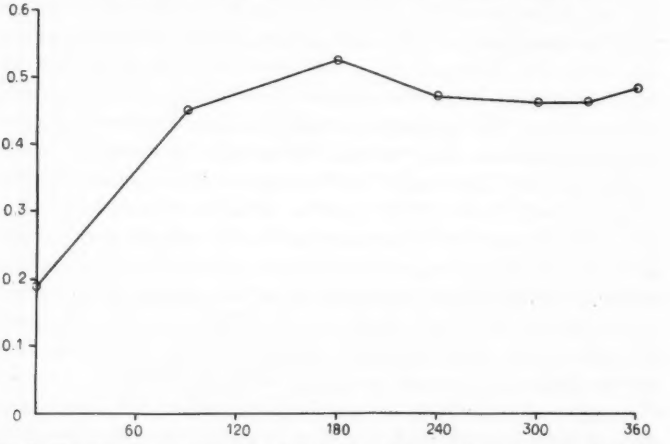

Fig. 6. The average concentration of ascorbic acid in the plasma of normal rabbits given 120 mg dehydro-ascorbic acid subcutaneously once hourly.

Abscissa: time in minutes after the injection of the first dose, ordinate: plasma concentration in mg/100 ml. Each point represents a mean taken from at least 5 rabbits.

level. In a few animals, however, the concentration of AA in the plasma rose in a similar manner to that observed in guinea-pigs and exceeded the concentration at which saturation occurs in the aqueous humour. These animals were discarded.

The plasma and aqueous values shown in Fig. 7 were recorded from a series of animals one hour after the last injection of DHA

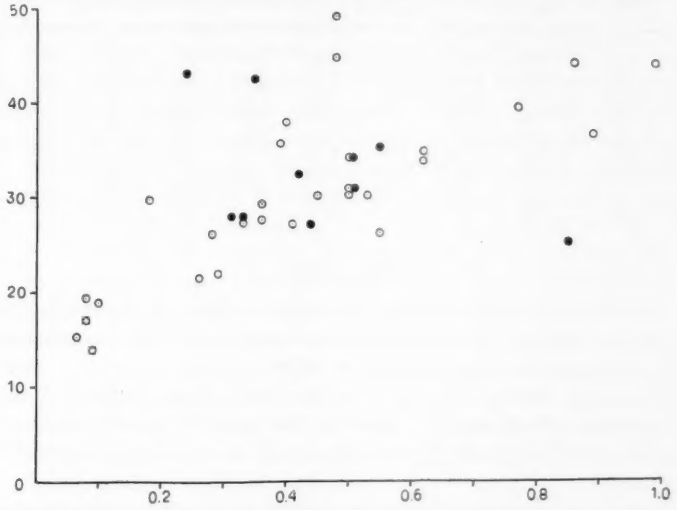

Fig. 7. The concentration of ascorbic acid in the aqueous humour of rabbits injected hourly with 120 mg of dehydro-ascorbic acid for five or six hours.

Abscissa: final plasma concentration of AA, ordinate: aqueous concentration, both in mg/100 ml. Each point represents the mean of the two eyes. Open circles are control animals, and filled in circles the injected animals.

and show that the concentration of AA in the aqueous humour does not differ markedly from that in control animals receiving no DHA and with a similar concentration of AA in the plasma.

Despite the known lability of DHA in the body an increased DHA/AA ratio in the plasma was found even one hour after the last injection in 6 out of 9 animals (Table 2) and it is therefore clear that the concentration of DHA in the plasma was increased at least periodically in these experiments. We have no corresponding data for the guinea-pigs but have every reason to believe that the injection of massive doses of DHA increased the DHA/AA ratio in this species too.

Table 2.

Steady state concentrations in plasma and aqueous humour (mg/100 ml) of rabbits after injection of DHA at hourly intervals. Column 5 indicates the plasma DHA predicted from the correlation found in normal plasma. The plasma samples were taken 1 hr. after the last injection.

Body weight kg	DHA injected mg/hr	Pl_{AA}	Pl_{DHA} Observed	Pl_{DHA} Calculated	Pl_{DKG}	Aq_1 T_1 min.	Aq_2 T_2 min.	$T_2 - T_1$ min.
2.2	120	0.51	0.50	0.18	2.98	16.1	30.8	360
2.2	120	0.55	0.29	0.19	3.95	15.6	35.1	360
2.8	60	0.85	0.12	0.23	1.20	18.6	25.0	312
2.2	60	0.31	0.06	0.15	0.27	19.6	27.9	307
2.4	120	0.35	0.33	0.16	1.44	21.9	42.4	279
2.6	120	0.24	0.76	0.14	2.89	16.2	43.1	295
2.7	120	0.42	0.36	0.17	2.39	16.7	32.3	307
2.0	120	2.07	0.67	0.40	3.79	15.8	65.8	305
2.2	120	2.39	0.42	0.44	3.44	22.5	87.5	309

Discussion.

The object of the present investigation has been to study the relation between the concentration of ascorbic acid in the aqueous humour and the blood plasma of rabbits and guinea-pigs in conditions of steady state and further, to determine whether the accumulation of ascorbic acid in the aqueous humour of these species is due to an uptake of dehydro-ascorbic acid from the plasma.

The concentration of ascorbic acid in the plasma of the guinea-pig found necessary to saturate the transport mechanism of the ciliary processes namely about 0.8 mg/100 ml is considerably lower than the 2 mg/100 ml. reported by LINNÉR (1952). Since however he used for the estimation of ascorbic acid the less specific 2,6 dichlorophenol-indophenol method this discrepancy cannot be considered significant. The plasma levels required to saturate the transport mechanism of the ciliary processes of rabbits confirm the observations of previous investigators (KINSEY 1947; LANGHAM 1950). However, although the maximal concentration of ascorbic acid in the aqueous humour of this species agrees with the observation of LANGHAM (1950) it is higher than that reported by KINSEY (1947) and LINNÉR (1952). In the experiments of KINSEY, insufficient time was allowed for conditions of steady state to be reached; the lack of agreement with the results of LINNÉR however cannot be due to this cause but may well reflect differences in breed of rabbits.

It is generally accepted that no synthesis of ascorbic acid occurs in the tissues of the guinea-pig and the lack of evidence for any synthesis of ascorbic acid in the eye adds further corroboration to this conclusion. In rabbits our results indicate the presence of a concentration of 14 mg ascorbic acid /100 ml. of aqueous humour when the concentration in the plasma approaches zero. Whether this is due to a synthesis of ascorbic acid by the lens or is due to some other cause cannot be decided from the present data.

Although the presence of dehydro-ascorbic acid in blood plasma has been a matter of dispute, recent work of STEWART, HORN and ROBSON (1953) leaves little doubt that it is contained in human plasma. The present experiments show that it also occurs in the plasma of rabbits. In guinea-pigs, where added ascorbic acid was not always fully recovered, the evidence is less convincing since the dehydro-ascorbic acid found could have arisen in vitro.

In the rabbit, the injection of dehydro-ascorbic acid resulted in no greater increase in the concentration of ascorbic acid in the aqueous humour than would be expected from the increase in the concentration of ascorbic acid in the plasma. For this species then, the present experiments have yielded no evidence that dehydro-ascorbic acid can be taken up by the eye and converted into ascorbic acid. The same conclusion was reached by KINSEY (1950) who conducted similar experiments but without attempting to establish steady state conditions.

In the guinea-pig, the concentration of ascorbic acid in the aqueous humour following injection of dehydro-ascorbic acid was actually less than anticipated from the ascorbic acid concentration in the plasma. The mathematical treatment used to compare the injected and normal animals is based on a number of perhaps questionable assumptions. Nevertheless, it would seem probable that the injection of large amounts of dehydro-ascorbic acid does cause a depression of the transfer mechanism responsible for the accumulation of ascorbic acid in the aqueous humour of this species.

Summary.

1. The part played by the ascorbic and dehydro-ascorbic acids of the plasma in determining the concentration of ascorbic acid in the aqueous humour of rabbits and guinea-pigs has been investigated using a modification of the Roe and Kuether method

for estimating ascorbic acid and its degradation products in the plasma.

2. When the concentration of ascorbic acid in the plasma approaches zero it disappears from the aqueous humour of guinea-pigs while in rabbits there remains a residue in the aqueous humour of about 14 mg/100 ml at the lowest plasma level observed.

3. With an increase in the concentration of ascorbic acid in the plasma, saturation of the transport mechanism of the ciliary processes occurs at about 0.8 mg/100 ml in guinea-pigs and at 2—3 mg/100 ml in rabbits. Above these plasma levels steady state concentrations of 24.6 ± 1.3 and 67.5 ± 2.1 mg/100 ml of ascorbic acid in the aqueous humour were observed in the two species.

4. The presence of a substance reacting like dehydro-ascorbic acid in the plasma of normal rabbits has been established, a low positive correlation ($r = 0.4$) existing between the concentrations in the plasma of ascorbic and dehydro-ascorbic acid.

5. Repeated subcutaneous injections of dehydro-ascorbic acid into rabbits and guinea-pigs did not increase the ascorbic acid concentration in the aqueous humour above that corresponding to the prevailing concentration of ascorbic acid in the plasma. In guinea-pigs evidence was found for a depression of the transfer mechanism by high concentrations of dehydro-ascorbic acid or its degradation products.

6. It is concluded that local synthesis of ascorbic acid in the eye does not occur in guinea-pigs but may exist in rabbits, and that the uptake of dehydro-ascorbic acid from the plasma by the ciliary processes is of little importance for the maintenance of the intraocular concentration of ascorbic acid.

This work was supported by grants from the British and Swedish Medical Research Councils. M.E.L. is indebted to the Royal College of Surgeons and Physicians for financial help from the Lister Award. Technical assistance was given by Miss S. Scotchbrook and Mrs. L. Väli. We wish to acknowledge the generosity of Roche Products in supplying samples of pure dehydro-ascorbic acid.

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From the Department of Zoophysiology, University of Lund, Sweden.

Occurrence and Variation of Arginine Phosphate during Insect Metamorphosis.

By

INGA PETTERSSON.

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During the years 1947 to 1952 AGRELL has performed several investigations concerning the metabolic changes during insect metamorphosis (see AGRELL 1952). In connection with these investigations the following work has been carried out in order to establish whether arginine phosphate is present in the insect pupa and in such case how its amount varies in relation to the developmental periods during metamorphosis.

Material and Methods.

The insect investigated was the fly *Calliphora erythrocephala* Meig. The imagines were given cane sugar, water, household yeast and raw horse meat, and the larvae as well as the pupae were kept at a temperature of 22°C $\pm$ 1°. The way in which the imagines, larvae and pupae were kept is thoroughly described by AGRELL (1947). The pupae which were collected 24 hours after all the larvae had become prepupae, were considered to have an average age of 12 hours. The age of $9^1/_2$ days was considered as 100 % development, as at this time half the sample of pupae had emerged. The age of 0 % development was the very short period — about one hour — when the larval skin had hardened but had not become brown. The arginine phosphate content was measured in prepupae, in pupae 0, 12, 24 hours old and then every day thereafter until 100 % development was attained and finally in newly hatched imagines.

The amount of arginine phosphate present in pupae can be measured if the "acid-soluble phosphate fraction" which contains phosphagen is isolated from the trichloracetic extracts of the pupae, and the arginine content of this fraction is determined colorimetrically after hydrolysis.

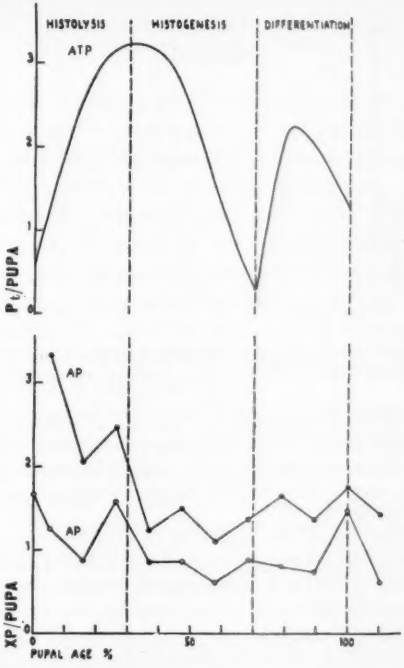

Fig. 1. The variation of adenosine triphosphate, ATP, (from AGRELL 1952) and arginine phosphate, AP, during metamorphosis. P_t = the terminal phosphate group of ATP.

The preparation of the extracts was carried out as follows: 20 pupae were washed and homogenated at 0°C and extracted in 3 ml 25 % trichloracetic acid for 2—3 minutes, an extraction method corresponding to that described by MEYERHOFER and LOHMANN (1928) when isolating arginine phosphate from *Astacus* muscle. The homogenate was mixed with solid barium hydroxide and brought to pH 9. The phosphates, whose barium salts are insoluble in water, were filtered away and the filtrate was made 60 % with regard to alcohol. In this alcoholic mixture the barium salts of some phosphates among them that of phosphagen, were precipitated (BALDWIN and NEEDHAM 1934). This precipitate was dissolved in water brought to pH 1—2 and the solution was then hydrolysed at 28°C over night. This hydrolysing method is said to be specific for arginine phosphate (BALDWIN and NEEDHAM 1934). Hydrolysis of the acidified extracts at 100°C for 15 minutes, however, gave values of arginine agreeing with those obtained when using the method first mentioned. For this reason the more rapid method was employed

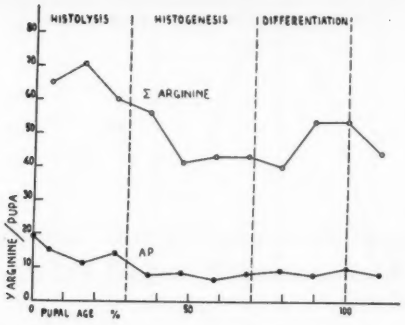

Fig. 2. The variation of the total amount of arginine in relation to that of phosphagen arginine. AP = arginine phosphate.

in most cases. The arginine content in the hydrolysed extracts was measured colorimetrically with Sackaguchi's method for the determination of arginine according to BLOCK and BOLLING (1948). The measurements were carried out in the Hilger spectrophotometer at the wavelength of 4,900 Å.

In order to determine the variation of arginine phosphate content during pupal development three complete series were carried out; two of these are seen in figure 1. Reproducible results were obtained and these values were further established by series comprising only a part of the developmental period.

In order to determine the total amount of arginine in pupae of different ages, the filtered trichloracetic acid extracts were hydrolysed at 100°C for 15 minutes, whereafter they were measured in Hilger spectrophotometer as described above. The results are shown in figure 2.

Attempts were made to determine the occurrence and variation of arginine phosphate during metamorphosis with the aid of *paper chromatography*. Alcoholic extracts were prepared from pupae of the same ages as those used for the colorimetric determinations. The variation in colour intensity of the spots on a chromatogram series should indicate the variation of arginine phosphate during metamorphosis. *The protein-free extracts* were obtained by homogenizing 20 pupae at 0°C and extracting the homogenate in 4 ml 80 % alcohol. The elution medium was n-butanol/glacial acetic acid and the paper was Munktells OB or Whatmann Paper No. 1. Arginine hydrochloride, in a concentration corresponding to 0.02-M arginine, dissolved in 80 % alcohol, was used as the test substance. From the pupal extracts an amount corresponding to 1 or 2 pupae was applied to each spot. From the test substance an amount corresponding to 40 γ arginine was applied to each spot. The chromatograms were developed by spraying them with Sackaguchi's reagent in order to obtain spots indicating arginine or, in order to obtain spots indicating phosphate, by spraying the papers according to HANES and ISHERWOOD (1949).

Results.

The curves in figure 1 show the variation in arginine phosphate during pupal development. The values are stated in γ P/pupa, P being calculated as the equivalent amount of arginine found. As phosphagens are very easily hydrolysed, possibly some part of the arginine phosphate may have been destroyed during the preparation of the extracts. As the errors thus obtained are systematic, we can still see from the curves in question the variation during development which was the aim of this investigation.

Figure 2 shows the variation of the total amount of arginine in relation to that of phosphagen arginine. In this figure the values are stated in γ arginine/pupa.

The results obtained by the use of the chromatographic method indicate too a variation in phosphagen content during metamorphosis. When developing the chromatograms with Sackaguchi's reagent, arginine was indicated by red spots. The pupal extracts gave two such spots, one at the same level as the test substance, and one between this and the initial spot. The "upper arginine spot" was much fainter than that below. The lower one could with certainty be considered as identical with the free arginine occurring in the pupal extracts; the upper one was thought to be identical with arginine phosphate from reasons which will be discussed in the following.

The arginine ran very slowly in the elution medium used and therefore no R_f-values could be obtained as the elution medium had to run through the paper several hours after the border had been reached. This was necessary in order to get the two arginine spots separated from each other.

The level of "the upper arginine spot" was calculated in relation to a yellow spot which was observed in each chromatogram of pupal extracts already before the paper had been dried.

The following relation was found:

$$\frac{l_{i-a_x}}{l_{i-p}} \quad \ldots\ldots\ldots\ldots\ldots\ldots (1)$$

l_{i-a_x} = the distance from the initial spot to "the upper arginine spot";

l_{i-p} = the distance from the initial spot to the "yellow spot".

The average value for this relation from 37 chromatograms developed in accordance with Sackaguchi was found to be 0.145 ± 0.004.

For the lower arginine spot on the same chromatograms the placement was stated in relation to the yellow spot, and the corresponding average number for the 37 chromatograms was found to be 0.48 ± 0.009. AGRELL (1949) found that the total amount of arginine in the pupa might occur as some easily hydrolysable compound.

When the chromatograms were developed according to HANES and ISHERWOOD for the detection of phosphate spots the following distance relation was determined:

$$\frac{l_{i-ph_x}}{l_{i-p}} \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (1')$$

l_{i-ph_x} = the distance from the initial spot supposed to be identical with the arginine phosphate.

The average number for relation (1') for the phosphate-developed chromatograms was found to be 0.143 ± 0.006.

The colour intensity of this phosphate spot indicating organically bound phosphate as well as that of "the upper arginine spot" showed a variation with the age of the pupae used for extracts, roughly agreeing with the variation of the arginine phosphate content found colorimetrically at corresponding ages. Thus for the following reasons "the upper arginine spot" should be identical with arginine phosphate: 1. Agreement in level of "the upper arginine spot" and one spot indicating organically bound phosphate. 2. The concurrent variation in colour intensity of the above-mentioned spots during pupal development. 3. The fact that an approximate agreement was found in the variation of colour intensity of the spots mentioned above and the measured amounts of "phosphagen arginine" in pupae of different stages of development.

Discussion.

In insect muscle as in every tissue containing arginine or creatine phosphate there is an enzymatic equilibrium according to LOHMANN (1936):

$$A - P \sim P \sim P + Ar(Cr) \rightleftharpoons A - P \sim P + Ar(Cr) \sim P,$$

where Ar = arginine

$\sim$ = "high energy bond".

Cr = creatine.

In processes where the energy connected with the terminal phosphate bond of ATP is called for, Lohmann's equilibrium will move to the left, and the phosphagen is used up.

AGRELL divides the metamorphosis of the fly Calliphora erythrocephala into three periods viz. 1. Histolysis from the entrance of pupation until 30 % of the total pupal age. 2. Histogenesis from 30 to 70 % development. 3. Differentiation from 70 % development until emerging. (AGRELL 1952.)

During the period of prevailing histolysis the catabolic processes preponderate over the anabolic ones, and for this reason the energy requirement of the pupa is diminished and the ATP content increases. Until 15 % development this increase in ATP seems to be partly dependent upon the fact that at the entrance of pupation the level of ATP is low and that of arginine phosphate is high. Lohmann's equilibrium will move to the left. As histolysis proceeds energy is accumulated in the energy-rich phosphate bonds of ATP, and Lohmann's reaction will again move to the right.

The period or predominating histogenesis signifies that the anabolic processes begin to outweigh the catabolic ones: the energy requirement of the pupa is increased and the energy accumulated as the energy-rich phosphate bonds of ATP is consumed. Of special interest is the decrease in arginine phosphate between 30 and 40 % of the developmental time. This decrease cannot be dependent upon muscular contractions as there are practically no muscles in the pupae during this time. The decrease in arginine phosphate is evidently dependent upon the fact that this energy reserve is required for the synthesizing processes. This is remarkable as the physiologic function of phosphagen is usually considered to be the formation of an energy reserve for only a few kinds of very intensive energy manifestations, viz. muscle contractions and discharges in the electrical organs of certain fishes. It is seen from figure 1 (AGRELL 1952) how ATP decreases at the same rate during the whole histogenesis; arginine phosphate on the other hand, will decrease at a slower rate than ATP does between 40 and 60 % of metamorphosis.

At the entrance of differentiation, when requirement for energy is again diminished, ATP increases rapidly again but arginine phosphate does not increase considerably until the last part of differentiation.

As the rôle of arginine phosphate in the imago is the formation of an energy reserve for muscle contractions we have to consider the variation of arginine phosphate during pupal development in relation to muscular growth during metamorphosis. This growth begins at 30 % of the total pupal age (AGRELL 1952) and continues until the end of histogenesis, but a considerable increase in phosphagen which is necessary for muscular contractions does not occur until 90 % of metamorphosis. Regarding the relation between muscular growth and a corresponding increase in those phosphate compounds which are necessary for contraction we may compare the insect metamorphosis with developing material of quite another kind, viz. muscle substance of chick embryos. HERRMANN (1952) found a rapid increase in the contractile substance of chick embryo muscles between the fourteenth and sixteenth days of incubation but concerning the phosphates necessary for contraction — ATP and phosphagen — there was no considerable increase until after the twentieth day of incubation. Thus in the insect pupa as well as in the chick embryo a considerable increase in muscular substance precedes that of phosphates necessary for contractions. As is seen from figure 1, however, the ATP curve falls between 90 and 100 % development, at the same time when the curve representing arginine phosphate rises. A decrease in the total amount of ATP in the pupa, however, does not exclude an increase in the ATP content of the pupal muscles. Actually this must be the case as according to Lohmann an increase in phosphagen is conditioned by an increase in ATP.

In the prepupae the phosphagen content lies at a lower level than in pupae at 0 % of metamorphosis. This must depend upon the cessation of muscular contractions as the prepupae become pupae. The imagines have a lower arginine phosphate content than pupae at 100 % of the developmental time. This is to be expected as very intensive muscular contractions occur when the pupae emerge.

The variation of arginine phosphate during metamorphosis shows a relation to that of the total amount of ATP in the pupa as long as up to 40 % of the developmental time. From this point of time, however, the arginine phosphate neither decreases during

histolysis nor increases during histogenesis at the same rate as does ATP. As long as there is practically no muscle substance Lohmann's reaction will hold for the whole pupa but this will not be the case when muscular substance has been formed.

The variation curve of arginine phosphate has the form of a shallow "U", a form approximately agreeing with that of the curve representing labile phosphate (AGRELL 1952, figure 17). The variation curve for arginine varies in the same way as the tissue pH during metamorphosis. This is in agreement with Lohmann, who found that phosphagen is synthesized from ATP when the pH is high. When the pH is low the reaction will be inverted. (LOHMANN 1936.)

Summary.

Arginine phosphate has been demonstrated not only in the imago but also in the prepupa and in the pupa of the fly Calliphora erythrocephala.

With the aid of paper chromatography and colorimetric determinations the relative amount of phosphagen arginine during different stages from prepupa to imago has been followed.

The variation found is discussed in relation to the energy metabolism during insect metamorphosis.

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From the Department of Zoophysiology, University of Lund, Sweden.

Changes in the Proteolytic Enzymes of the Yolk in the Developing Hen's Egg.

By

HADAR EMANUELSSON.

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As is well known, in an early stage of the development of the chick embryo there is a separation of the embryo from the yolk and the embryo from then on is totally dependent for its nutrition on the yolk sac, through which all absorption of yolk and white is effected. The general opinion is that absorption is arranged so that endodermal cells of the yolk sac secrete digestive enzymes into the yolk and the cells then absorb the liquefied material and continue its digestion. Through the circulating blood the products are then carried to all parts of the growing embryo.

For general surveys of enzymes in the hen's egg prior to and during incubation the reader is referred to papers by LINEWEAVER et al. (1948) and NEEDHAM (1931, 1950).

Investigations on proteolysis in the yolk of the hen's egg have been made by several scientists, BORGER and PETERS (1933), GOLDSTEIN (1938), MYSTKOWSKI (1936), REMOTTI (1926) and others. The most important of these investigations is REMOTTI's in which the physiology and the histology of the yolk sac are thoroughly discussed.

The Italian scientist has arrived at the result that proteolytic activity in the yolk is low during the first days of the incubation. After 6 days' incubation the activity is greatly increased and the maximum is reached on the ninth day, whereupon the activity remains at the same level until hatching.

REMOTTI's method for measuring the proteolytic activity has been formol titration according to SØRENSEN, and consequently his figures are a measure of the number of carboxyl groups liberated during the hydrolysis. Naturally the result is a measure of total protein hydrolysis, but it gives no information concerning the number of enzymes functioning or their characteristics.

As the present author has been able to show in an earlier publication (EMANUELSSON 1951) that free amino acids as well as three different proteolytic enzymes appear in the yolk of the hen's egg prior to incubation it was then decided to follow the proteolysis in the yolk during incubation.

The object of the investigation has been partly to study possible quantitative and qualitative changes occurring in the amino acids and partly to characterize the proteolytic enzymes present.

Materials and Methods.

For the investigation eggs from a pure breed of White Leghorn have been used. The eggs were incubated in an egg-incubator at 38° C.

From the beginning the author's intention was to take samples from different parts of the yolk, as REMOTTI had been able to show that a certain gradient in proteolytic activity occurs in the yolk.

In the sampling of the yolk it appeared, however, that it was very difficult to obtain material which was with absolute certainty localized to a fixed part of the yolk.

Prior to incubation the yolk has a very homogeneous appearance, but after a few days' incubation this condition becomes changed through uptake of water from the white. Later on the vitelline membrane grows weaker and one has to be extremely careful in handling the yolk in order to keep the membrane intact.

Attempts to obtain the preparation more easily through freezing the yolks failed. A stratification, obviously artificial, was obtained in this way and precluded the possibility of obtaining reliable results by this method.

Instead the samples had to represent the whole yolk: after removal of the embryo as much as possible of the contents of the yolk was collected. In order to get a sample as representative as possible several yolks (10—20) were used for every experiment.

Investigations of amino acids.

Analyses of amino acids — which were made at the same time as the analyses of the proteolytic activity — had the following course: the samples were precipitated with 96 per cent alcohol to a final concentration of 80 per cent. Then they were homogenized and the pre-

cipitated proteins were filtered off. The alcoholic solution that was obtained was chromatographed one dimensionally, sometimes also two dimensionally. Munktell OB paper was employed for the chromatograms. Solvent: butanol — acetic acid — water according to PARTRIDGE (1948).

Investigations of proteolytic enzymes.

Samples of yolk were homogenized together with ice-cold 30 per cent glycerol solution, which proved to be a suitable extractive agent. The homogeneous solution was then centrifuged. The supernatant, with a pH of about 6.5 was used for measuring the proteolytic activity. Principally this was performed according to NORTHROP et al. (1948, p. 307).

In the procedure the solution is first buffered with Mc Ilvaine's standard buffer to the desired pH, then the enzyme-substratum, 1 per cent hemoglobin solution (Homo), is added, and finally the test-tube with the mixed solutions is kept in a water-bath at exactly 37° C for 2 or 3 hours. Preliminary experiments showed that the proteolysis had a rectilinear course at least for the first 4 hours.

When the test-tubes were kept in water-bath they were constantly stirred in order to prevent any sedimentation in the solution. After addition of buffer to the samples a faint precipitation was observed in those where the pH was about 5, obviously the isoelectric point for certain proteins in the samples. In order to eliminate such precipitation in the samples, all of them had to stand at 0° C for 15 minutes after addition of the buffer, then the samples were filtered immediately before the test-tubes were placed in the water-bath. Preliminary experiments showed that such a procedure did not interfere with the enzymatic conditions in the samples.

The enzymatic activity was interrupted through adding strong trichloracetic acid or perchloric acid to the samples. The acid precipitated all proteins but left amino acids liberated during the proteolysis. Of the amino acids the cyclical, with absorption in ultraviolet at 2,750 Å were measured in a Hilger spectrophotometer.

In the measurement there was a drawback with the trichloracetic acid: because of its own strong absorption in ultraviolet, the sensitivity of the measurements was lowered. In experiments employing perchloric acid instead of trichloracetic acid this disadvantage was eliminated, but here the filtrations were more difficult to perform because of the more finely dispersed precipitate that was obtained.

Addition of strong acid to the samples fixed their pH and thus variations in absorption (for tyrosine) with pH were eliminated.

All experiments were carried out in duplicate and good agreement was obtained.

In order to investigate the spontaneous activity in the samples, identical series were run that differed only in the enzyme substratum, the hemoglobin solution was here exchanged for distilled water.

Attempts to follow the proteolysis through applying viscosimetrical

analyses to the yolk itself failed, as it was evident that the method could be used only for very diluted yolk-solutions.

In some experiments cysteine hydrochloride wad added as an activator to the samples. The cysteine hydrochloride was added as a neutralized solution to a final concentration of about 100 mg per cent.

Results.

Amino acids.

Qualitative changes in the alcoholic extracts from yolk from various states of incubation could not be demonstrated for amino acids or for alcohol-soluble peptides. (See EMANUELSSON 1951.)

Quantitatively a weak increase in concentration of the amino acids was noted after some days' incubation (estimated by comparing colour intensity of spots in chromatograms from comparable samples). As the increase seemed to be comparatively small, maximally 50 per cent, it was not investigated further.

Proteolytic enzymes.

The investigation shows that the proteolytic activity in the yolk during incubation is distributed among at least three different protein-splitting enzymes with quite different pH-optima. These are: No. 1. Enzyme with optimum about pH = 3.

No. 2. » » » » pH = 5.

No. 3. » » » above pH = 7.

The optima correspond well to those that were obtained for the enzymes appearing in the yolk of the hen's egg prior to incubation (EMANUELSSON 1951), and therefore it is assumed that the enzymes are identical.

Enzyme No. 1.

For calculating the activity of this enzyme the amount of amino acids (tyrosine) liberated at optimum pH was measured.

The activity, measured after the samples had been kept in a water-bath for 2 hours is relatively low: enzyme from 1 cc yolk from an unincubated egg gives in that time about 0.3 mg liberated amino acid (estimated as tyrosine). When incubation of the eggs has begun the activity per cc yolk is increased (see fig. 1). On the second day of incubation the activity amounts to more than double the value for the unincubated egg. Further increase could

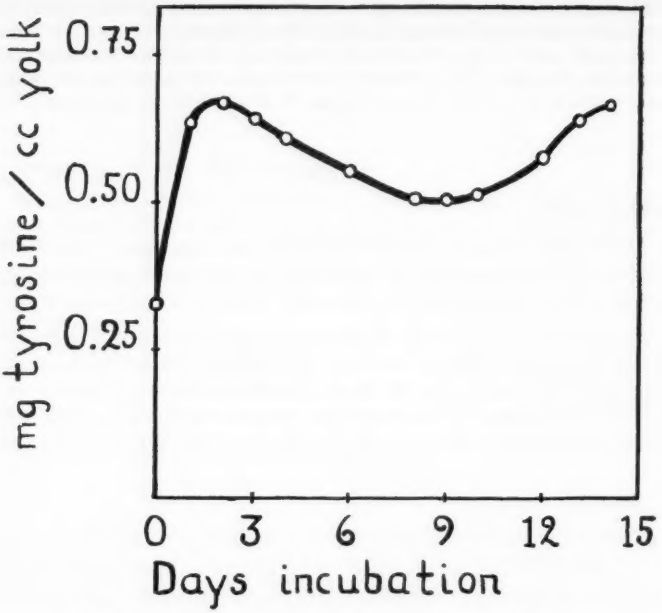

Fig. 1. Proteolytic activity of enzyme No. 1 in yolk.

not be noted, on the contrary, a small decrease was observed, but it was only of short duration.

When calculating the activity the dilution of the yolk during incubation has not been accounted for. However the results are not appreciably changed, when this is taken into consideration (see ROMANOFF 1933).

Experiments have been made to measure possible changes in the pH in the yolk during incubation of the eggs. Only small differences (± 0.5 pH) could be found however. The existence of special loci in the yolk that had another pH than the surrounding area could not be proved.

In connection with the investigations on proteolytic activity in the yolk, the activity in the homogenized yolk-sac was also studied. The investigated material was taken from eggs that had been incubated for 10—21 days.

It appeared that enzyme No. 1 was present here, but its activity was of quite another magnitude: after 10 days' incubation

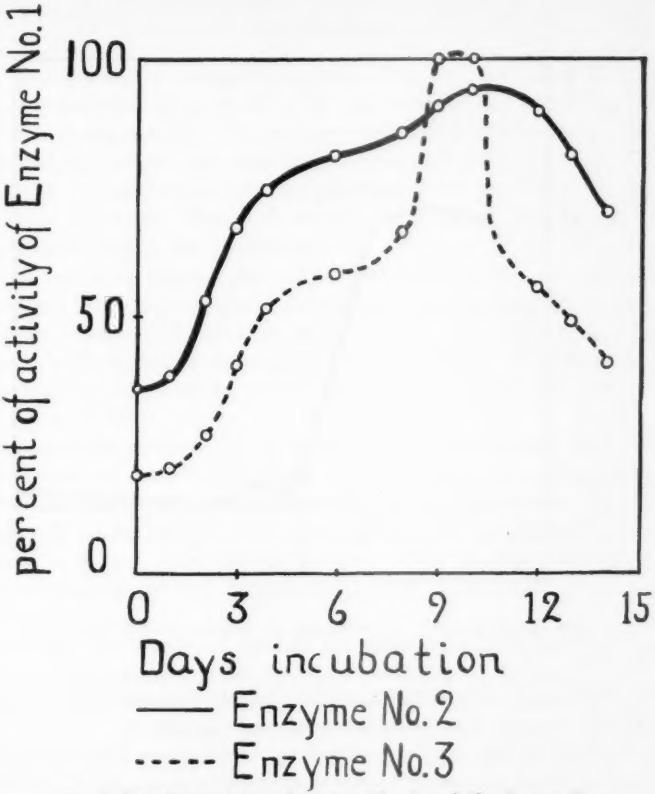

Fig. 2. Relative activity of enzyme No. 2 and No. 3 in yolk.

the activity in the yolk-sac was about 10 times that in the yolk. The activity in the yolk-sac remained at this level until hatching. See figure 3.

Enzyme No. 2 and Enzyme No. 3.

As a measure of their activity the amounts of amino acids (tyrosine), liberated at pH 5 and pH 7.5 were estimated.

For enzyme No. 3 no pH-optimum was obtained, as previously stated, a well-known fact for tryptical enzymes (NORTHROP et al. 1948). As the slope of the pH-curve is slight and as measurements have shown that the pH in the samples is constant during

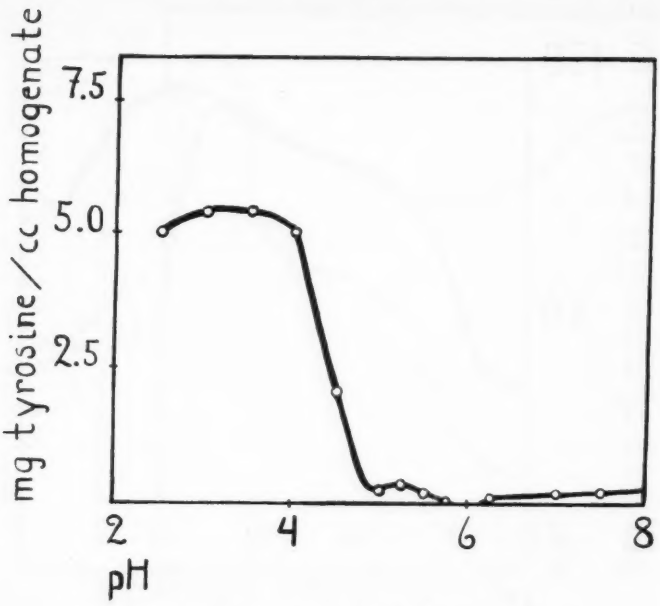

Fig. 3. pH-Activity of proteinases in yolk-sac homogenate. Incubation: 20 days.

the incubation, it will be correct to let the measurements from pH = 7.5 stand for the activity of enzyme No. 3.

In figure 2 the absolute values are not reported but instead relative values compared to the activity of enzyme No. 1 at the same state of incubation.

It appears that there is a rapid increase in the activities of enzymes No. 2 and No. 3, both relatively and absolutely, and maxima are reached for both about the ninth day of incubation. Later a small decrease is noted.

Experiments in which samples were activated with cysteine hydrochloride were made on yolk from eggs that had been incubated a few (0—3) days. It was found that enzyme No. 2 but not Nos. 1 and 3 could be activated in this way 2—3 times.

As to the yolk-sac, the presence of enzyme Nos. 2 and 3 could be established, even if their activities, compared with that of enzyme No. 1 were low. The activities remained at the same low level until hatching. See figure 3.

Discussion.

The present investigation agrees with the prevalent opinion concerning the decomposition of the yolk in the hen's egg. Of the enzymes studied, at least one (No. 1) has with very great probability leaked out from the embryo and its membranes, but for the others there is also the possibility that they may be pre-formed enzymes, deposited in the yolk, which are gradually activated during the incubation.

In this investigation proteolytic activities in the yolk and the yolk-sac have been studied, but as only very preliminary experiments on proteolytic activity of the embryo-body itself have been made with the methods employed for the yolk, a discussion concerning the origin of the proteolytic enzymes in the yolk will not be very fruitful.

Concerning enzyme No. 1 however, it can be said that the difference in activity between the yolk and the surrounding yolk-sac makes it very probable that an enzyme of this type in the yolk comes directly from the yolk-sac through diffusion. The gradient found, between yolk-sac and yolk is thus in good agreement with the general opinion about yolk-utilization in the hen's egg.

As to enzymes No. 2 and No. 3 there is no such gradient between yolk-sac and yolk. As has already been stated only very preliminary experiments on proteolytic activity of the embryo-body have been made. From these it seems that in homogenized 1-day-old embryos activity of proteolytic enzymes at pH 5 and pH 7.5 is lower than in yolk from the same eggs. This would indicate another origin of these enzymes than the embryo and speaks in favour of a liberation of pre-formed enzymes.

The low activity of the three enzymes in the yolk speaks as much in favour of diffusion from the embryo as of successive activation of pre-formed ones. In this connection it ought to be mentioned that the low activities must not be ascribed to the fact that hemoglobin is an unnatural substratum, as the pH-curves representing the spontaneous activities in yolk samples from the various stages of incubation have essentially the same shape as those calculated for activity upon hemoglobin alone. The activities are in both cases of the same order of magnitude.

The fact that enzymes occurring in the yolk of the incubated hen's egg already appear in the unincubated egg might perhaps

indicate a deposition of pre-formed enzymes. However, it must not be forgotten that even in new-laid eggs the embryos have already begun their development and form distinct blastoderms. For that reason too much importance must not be attached to this fact.

The possibility of activating enzyme No. 2 offers no definite information concerning the origin of the enzyme. Even if the possibility of activation makes a pre-formation probable, MYSTKOWSKI's (1936) information about cathepsin in the chick embryo, which can be activated with cysteine hydrochloride in the same scale, suggests identity between enzyme No. 2 and cathepsin and the embryo-body as the probable site of origin of the enzyme.

From the foregoing there is no definite support for one theory or the other as to the origin of the two enzymes. Results up to now, however, seem to speak more in favour of a pre-formation than of a diffusion from the embryo.

The existence of a gradient between yolk-sac and yolk in regard to enzyme No. 1 emphasizes the necessity of performing the sampling very critically. Especially in more advanced stages great caution must be taken so that no blood-vessel is ruptured thereby contaminating the sample.

From the investigation it appears that, during the part of incubation of the hen's egg that has been studied (0—15 days), there are distinct maxima in activity for enzymes Nos. 2 and 3 after about 9 days' incubation. For enzyme No. 1 no maximum can be demonstrated at that time.

It is of interest that this maximum after 9 days' incubation coincides with the results obtained by REMOTTI (1926). He has found with his method a proteolytic maximum at that time.

NEEDHAM (1931) has emphasized that the protein catabolism of the chick embryo, expressed as catabolised protein per g dry-weight, has a maximum at 9 days' incubation and this coincidence makes it probable that there is a connection between the protein catabolism of the embryo-body and the enzymes discussed, Nos. 2 and 3.

Further evidence indicating the importance of the proteolysis in the 9-day embryo is that embryo-extract with optimal effect upon tissue culture is obtained from the 9-day stage and those proximal thereto.

As to the amino acids, WILLIAMS et al. have stated in a recently published paper (1954) that they have studied the free amino acids in the yolk during incubation. With their methods, using ion-exchangers and measuring the optical densities of the acids, they found for some amino acids an increase from 2—10 times from the third to the ninth day of incubation. A general maximum seemed to be localized to the ninth day of incubation. In the author's opinion the greater increase found by WILLIAMS et al. may perhaps be ascribed to hydrolysis during preparation.

Even if our knowledge concerning intermediate products in protein metabolism is very limited, it is, nevertheless, improbable that in a protein catabolism followed by anabolism, as in the hen's egg, the process should be limited to amino acids. Probably there must be an equilibrium in which the amino acids have a restricted part only and in which more or less complex peptides dominate.

Similar results as to the amino acids are reported from the pupae of the fly *Calliphora* (AGRELL 1951). In the pupae, which form an isolated system comparable to the hen's egg there is a steady level during the whole metamorphosis period in spite of the fundamental transformation of larva-protein into imago-protein which takes place here.

Recent statements (SCHECHTMAN and KNIGHT 1954) about the ability of the ovum of the chicken to incorporate proteins directly without any previous decomposition thereof makes it necessary for us to revise earlier conceptions of protein metabolism. Perhaps direct protein transfer provides part of the proteins of the young embryo.

Attempts to isolate the intermediate products in the protein metabolism of the chick embryo have been made by the author and are in progress. In these paper chromatographic and electrophoretic methods have been employed.

With the paper chromatograms, for which strong buffer solutions have been used together with impregnated papers of various types, no valid results have been obtained.

Electrophoresis in filter paper, however, has given promising results and this is also true of electrophoresis in starch.

Summarizing it can be said that the present investigation emphasizes the central position held by the 9-day stage in protein metabolism during the development of the chick embryo.

As the hen's egg must be considered a very suitable object for

studying the general pathways in protein metabolism it is to be anticipated that further investigations will be focussed just to the 9-day stage.

The investigation has been facilitated by grants from the Kungliga Fysiografiska Sällskapet, Lund, and from the Swedish Research Council for Natural Science.

Summary.

Free amino acids and alcohol-soluble peptides in the yolk of the hen's egg have been studied during the first 14 days of incubation. No qualitative and only small quantitative changes could be noticed. During incubation three different proteolytic enzymes occur in the yolk and in the yolk-sac. In the yolk maxima for these enzymes were found about the second and the ninth day of incubation. Yolk-protein-utilization of the embryo is discussed.

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From the Thunberg Laboratory of the Institute of Physiology, University of Lund, Sweden.

The Influence of the Adrenal Cortex on the Citric Acid Concentration of the Guinea Pig's Blood Serum.

By

INGA-GRETA AGRELL, S.-E. LINDELL and H. WESTLING.

Received 15 January 1955.

The influence of the adrenal cortex on citric acid metabolism has received little attention. Observations of increased citric acid concentration in blood serum from patients with Addison's disease (MÅRTENSSON 1949) indicate, however, that a closer study of the influence of the adrenal cortex on the metabolism of citric acid might be valuable.

Experimental.

Male guinea pigs weighing 300—500 grams have been used. This species was chosen because its adrenals are large and easy to identify. Besides, accessory cortical tissue is rare in guinea pigs (KAHLSON, LINDELL and WESTLING 1953). The animals were kept under uniform conditions and were given food consisting of beets and hay ad libitum.

Blood samples, 1—2 ml, were taken by heart puncture after 6—7 hours of fasting. Anaesthesia was given with ether, which has been shown by MÅRTENSSON (1943) not to influence the citric acid content of rabbit's serum. The heart punctures were performed between 15.00 and 16.00 p.m. with intervals of 1—2 weeks. The heart puncture was the immediate cause of death in about 10 % of the animals. In order to obtain an approximate value of the hemoconcentration after adrenalectomy, the number of erythrocytes per mm^3 of blood was counted in 25 guinea pigs before and after operation. The blood obtained by heart puncture was used.

Removal of the adrenal glands was performed in two stages with 3—5 weeks' interval, starting with the right gland as described by KAHLSON, LINDELL and WESTLING (1953). The same precautions as regards remnants of the adrenals were observed. Thus the present series consists only of animals in which the adrenals had been removed intact within their capsules and where a careful post mortem examination did not show any remnants or accessory adrenal glands.

To investigate the effects of the operative trauma 13 animals were subjected to sham operations. In these animals a left dorsal incision was made and the region of the left adrenal was exposed, the adrenal gland being left intact.

After removal of the second adrenal and after sham operation the animals were kept in a special, heated cage under close observation. They were weighed twice daily.

17-hydroxy-11-dehydro-corticosterone acetate in crystal suspension, Cortisone acetate Ciba (cortisone), or desoxy-corticosterone acetate in oil solution, Percorten Ciba (DCA), were injected intramuscularly twice daily, 5 mg each time.

Citric acid determinations were performed with the enzymatic methylene blue method of THUNBERG (1929, 1933) as modified by MÅRTENSSON (1940). As a rule duplicate determinations were made.

Results.

The citric acid content in microgram per ml of serum (ci) in 33 normal guinea pigs was found to be 108.3 ± 1.9 (79 samples). The mean value observed in 21 animals studied 3—6 weeks after removal of one adrenal was 98.4 ± 1.9 (43 samples), the difference from the normal value being significant ($p < 0.05$). After removal of the second adrenal in the latter animals a progressive increase in ci ensued (table 1). The mean value after 2 days was 129.5 ± 14.8 (11 animals), after 3—5 days 142.4 ± 12.3 (11 animals) and after 6—11 days 174.7 ± 12.9 (6 animals). The mean per cent increases in the different groups are shown in table 1. The increases are statistically significant.

In animals subjected to sham operation low serum citrate values were observed (table 1), the reduction being statistically significant 2—6 days after the operation. A gradual return to normal values occurred, the preoperative value being reached after 2—4 weeks (table 1).

After removal of both adrenal glands the animals lost progressively in weight (table 1). In the individual animal there was no parallelism between the weight loss and the increase in ci. Thus, one animal had a 10 % decrease in body weight two days after

Table 1.

Changes in ci and body weight in the different groups.

Operation	Treatment	No. of animals	Days after op.	Per cent change in	
				ci (± S.E.M.)	body weight
Adrenalectomy	—	11	2	+ 32 (± 14)	— 12
»	—	11	3— 5	+ 49 (± 13)	— 15
»	—	6	6—12	+ 67 (± 10)	— 18
»	Cortisone	5	2— 7	+ 1 (± 4)	— 6
»	DCA	4	3— 7	— 3 (± 4)	— 9
Sham operation	—	6	2	— 16 (± 3)	— 15
»	—	7	3— 6	— 12 (± 3)	— 6
»	—	8	9—10	— 6 (± 16)	— 7
»	—	8	16—21	— 3 (± 5)	— 1

adrenalectomy, when the ci was found to be 88 % higher than the preoperative value. Another animal had lost 16 % in body weight two days after adrenalectomy and its ci had increased only 2 %. Five days after removal of the adrenals one animal had a ci increased of 51 % with an 11 % weight loss, whereas another animal of the same size had lost 12 % in body weight with no change in ci five days after adrenalectomy. The correlation coefficient between weight loss and ci increase in all animals was 0.31, *i. e.* no correlation was demonstrable.

The mean value for red blood cells in normal guinea pigs was found to be 5.5 millions per mm^3 of blood. After adrenalectomy there was an increase in the erythrocyte count in most of the animals, but the values showed a great variation, probably due to varying degrees of bleeding at the operation and the punctures. There was, however, no parallelism between the increase in ci and the hemoconcentration as measured by red blood cell count. In one animal, for example, with a 12 % increase in erythrocyte count 5 days after adrenalectomy the ci was not elevated, whereas it had increased 51 % in another animal, which at the same time after adrenalectomy had a 14 % increase in erythrocyte count. Sham operation produced no significant change in erythrocyte counts.

Treatment with cortisone, 5 animals, or DCA, 4 animals, prevented the increase in ci after adrenalectomy. (Table 1.) After withdrawal of the steroid injections the ci increased as in adrenalectomized animals not treated with steroids. It was also found that administration of cortisone (6 experiments in 4 animals)

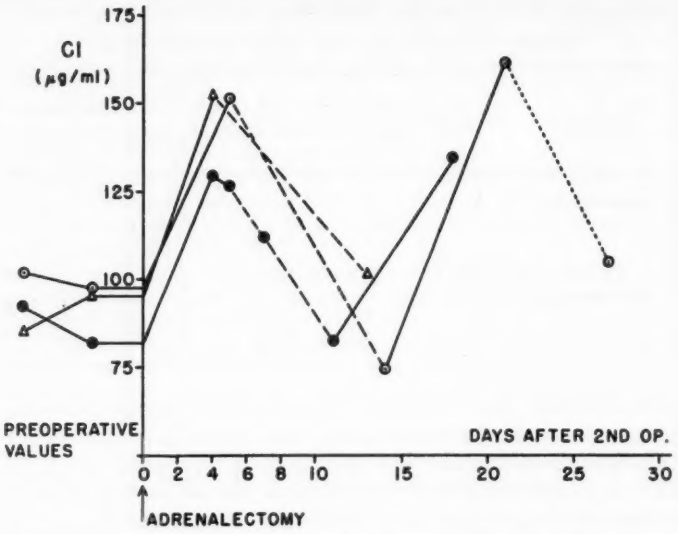

Fig. 1. Citric acid content of serum (µg/ml) in three guinea pigs after adrenalectomy and hormone injections.
Cortisone treatment = interrupted lines. DCA treatment = dotted lines.

or DCA (1 experiment) to adrenalectomized animals with increased ci resulted in a lowering of the ci to the initial value (Fig. 1). The return to normal seemed to take 2—3 days.

Discussion.

In guinea pigs, after removal of the adrenal glands, an increase of the citric acid content in blood serum was found. This was not due to the unspecific operative trauma, since animals subjected to sham operation showed values lower than normal. Moreover, administration of cortisone or DCA prevented the hypercitricemia and if given to an adrenalectomized animal with increased serum citrate caused a return to normal level. This indicates that the rise in serum citric acid is due to loss of the adrenal cortex.

With the dosage employed, no difference was observed between the two steroids as regards ci. However, only one value has been obtained after administration of DCA to adrenalectomized animals with increased ci. The reason for this is that the other animals

with increased ci, which received DCA, succumbed before another sample could be obtained.

The increased serum citrate after adrenalectomy is in agreement with MÅRTENSSON's (1949) observations in patients with Addison's disease.

The cause of the hypercitricemia in adrenal insufficiency is not known. The increased concentration of serum citrate could simply be due to the hemoconcentration that follows adrenalectomy. The lack of correlation in our experiments between weight loss, hemoconcentration and increase in serum citrate indicates, however, that other mechanisms are involved. The increased serum citrate could for instance be secondary to decreased hepatic or renal function, conditions in which hypercitricemia may occur (SJÖSTRÖM 1937, MÅRTENSSON 1940).

The most likely explanation, however, is that the increased serum citrate reflects the changes in intermediary metabolism in adrenal insufficiency, since the "citric acid cycle" is considered to be the principal pathway for the final oxidation of 2-carbon fragments, whether these arise from carbohydrate, protein or fatty acids. (HAWK, OSER and SUMMERSON 1954.)

Sham operations were followed by a decrease in ci lasting 2—4 weeks. It is interesting to note that this was the usual time necessary for complete healing of the operative wounds. A similar reduction of ci has been observed in human beings after surgical operations, infections and in thrombosis (SCHERSTÉN 1931, SJÖSTRÖM 1937). It is tempting to regard the hypocitricemia in these conditions as part of the metabolic changes that follow trauma and repair, possibly mediated via the adrenal cortex. The fact that the unilaterally adrenalectomized animals had lower ci values than the normal guinea pigs might be explained in a similar way.

Summary.

1. In guinea pigs adrenalectomy was followed by a significant increase in the citric acid content of blood serum, determined with Thunberg's enzymatic methylene blue method.

2. This increase could be prevented by cortisone or DCA.

3. After sham operations subnormal serum citrate values were found.

Skilful technical assistance was given by Miss Kerstin Anderson, Miss Edith Lehmann and Mrs. Asta Lundqvist. Mr. N. Sandberg prepared the figure.

Part of the expenses of this investigation were defrayed by grants from the Nordisk Insulinfond. Ciba Produkter AB, Stockholm, supplied us with the steroids.

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From the Department of Biochemistry and Nutrition, Polytechnic Institute, Copenhagen.

Influence of Various Levels of Dietary Cholesterol on the Cholesterol Content of Certain Organs and of Bile of Chicks Fed Fat-free and Peanut Oil Containing Diets.

By

HENRIK DAM, INGE PRANGE and EBBE SØNDERGAARD.

Received 2 February 1955.

The purpose of the present study was to determine approximately the level of dietary cholesterol beyond which there is accumulation of cholesterol in liver, spleen, aorta and bile of growing chicks fed an experimental diet without fat or with 10 % peanut oil.

An experimental period of four weeks was chosen, commencing about 2 weeks after hatching at which time the chick has disposed of the main part of the cholesterol surplus it has taken over from the egg (DAM 1931). After this initial period synthesis of cholesterol in the chick exceeds its excretion and breakdown, if cholesterol is not supplied in the diet (DAM 1929).

The basal diet was one that gave fairly good though not optimal growth. It contained slightly less than the amount of choline (700 mg per lb.) indicated in Recommended Nutrient Allowances for Poultry (1946), and a relatively high amount of protein, though not of optimal composition for chicks since growth can be improved by increasing the content of casein or by addition of cystine.

Experimental.

Day-old chicks (cross-bred of white leghorn and light sussex) were reared on a commercial diet (DAM and SØNDERGAARD 1953) for 13 days whereafter they were transferred to one of the diets presented in table 1

Table 1.

Composition of basal diets.

Diet no.	1	2	3	4
Casein, "vitamin test"[1]	200	200		
Casein, crude[2]			200	200
Gelatine	80	80	80	80
Salt mixture[3]	51.7	51.7	51.7	51.7
Vitamin mixture[4]	1.0	1.0	1.0	1.0
Choline chloride	1.5	1.5	1.5	1.5
Sucrose	665.8	565.8	665.8	565.8
Peanut oil		100		100
	1,000	1,000	1,000	1,000

All diets were supplemented with 10 mg Synkavit (Roche)[5] and 100 mg Ephynal (Roche)[6] per kg.

Vitamins A and D were given as concentrates[7] dissolved in Filtrol-treated olive oil, 1 drop twice a week per animal. This corresponds to 220 I. U. A and 20 I. U. (chick) D per day.

[1] From Genatosan, Ltd., Loughborough, England, containing 0.11 % fat.
[2] "Dairinex", from A/S Dansk Mejeri Industri & Export Kompagni, Stege, Denmark, containing 1.2 % fat.
[3] DAM and SØNDERGAARD 1953.
[4] DAM and SØNDERGAARD 1953.
[5] Di-calcium-salt of 2-methyl-1,4-naphthohydroquinone-diphosphoric acid ester.
[6] d,l-α-tocopherol acetate.
[7] Made up from vitamin A concentrate, natural ester form, containing 838,000 I. U. A per g 1.7 g; vitamin D_3 concentrate containing 200,000 I. U. (chick) per g (in Filtrol-treated olive oil) 0.63 g; Filtrol-treated olive oil 47.67 g, total 50 g. The vitamin A concentrate was kindly furnished by Distillation Products Industries, Rochester, N. Y.

with or without addition of cholesterol in the amounts listed in tables 2 and 3. For diets with 10 % peanut oil the cholesterol was dissolved in the oil before being incorporated into the diet. Amounts of cholesterol exceeding 0.33 % of the diet could not be kept in solution in the oil at room temperature.

The animals were sacrificed after 4 weeks' feeding of the experimental diets.

Liver, spleen, aorta (from the right brachio-cephalic to the renal arteries) and bile from the bladder were taken for determination of total cholesterol.

Analytical Procedure.

Liver and spleen were heated on a boiling waterbath for two hours with aqueous 30 % KOH and treated further as in earlier studies on muscles (DAM, PRANGE and SØNDERGAARD 1952) using the Liebermann-Burchard reaction.

Aortae were dried over P_2O_5 *in vacuo* and heated with 5 ml of 30 % aqueous KOH and 2 ml of absolute alcohol for two hours on boiling

Table 2.

Influence of dietary cholesterol and peanut oil on liver cholesterol of chicks. Feeding period 4 weeks.

Group no.	Basal diet no.	Type of casein	Peanut oil %	Addition to basal diet: cholesterol %	Average weight of animals[1] g		Mg cholesterol per 100 g liver[2] (wet weight)
					initial	final	
1241	2	"vitamin test"	10	0	83^{10}	263^{9}	$290^{8} \pm 9$
1242	2	»	10	0.01	83^{10}	246^{9}	$304^{8} \pm 11$
1243	2	»	10	0.033	83^{10}	245^{9}	$298^{8} \pm 8$
1244	2	»	10	0.1	83^{10}	239^{10}	$331^{8} \pm 29$
1245	2	»	10	0.33	83^{10}	290^{10}	$1{,}182^{8} \pm 94$
1246	2	»	10	1.0	83^{10}	256^{10}	$2{,}196^{8} \pm 303$
1247	2	»	10	3.3	83^{10}	304^{10}	$4{,}416^{8} \pm 131$
1248	1	»	0	0	83^{10}	190^{10}	$485^{9} \pm 19$
1249	1	»	0	0.1	83^{10}	228^{9}	$491^{9} \pm 25$
1250	1	»	0	1.0	83^{10}	181^{10}	$543^{9} \pm 20$
1251	4	crude	10	0	83^{10}	275^{10}	$313^{8} \pm 19$
1252	4	»	10	0.1	83^{10}	293^{9}	$310^{8} \pm 17$
1253	4	»	10	1.0	83^{10}	305^{9}	$2{,}343^{8} \pm 170$

[1] The superscripts represent number of chicks in the groups.
[2] The superscripts represent the number of livers assayed; ± indicates standard error of the mean.

waterbath; after addition of 5 ml of water, the non-saponifiable material was extracted 3 times with petroleum ether, washed, evaporated to dryness, and treated as above. Three or four aortae were taken together for one determination.

Bile (0.05—0.3 ml) was heated for 2—3 hours on a boiling water bath with 4 ml of 17 % KOH in 50 % alcohol, after which 2.5 ml H_2O was added and the non-saponifiable fraction shaken out three times with 5 ml petroleum ether, washed, evaporated to dryness, dissolved in chloroform and cholesterol determined by the more sensitive color reaction with acetylchloride and zinc chloride (528 mμ) (modification of S. GÖRTZ (1934), and (1937)).

Determination of fat in the two caseins was carried out by the method of SCHMID, BONDZYNSKI and RATZLAFF as described by FLEISCHMANN-WEIGMANN (1932).

Results and Discussion.

The results are presented in tables 2 and 3, and fig. 1.

1. Diets with 10 % peanut oil:

No significant increase of cholesterol is observed in liver, spleen, aorta and bile when the cholesterol content of the diet varied

Table

Influence of dietary cholesterol and peanut oil on cholesterol in

Group no.	Basal diet no.	Type of casein	Peanut oil %	Addition to basal diet: cholesterol %	Average weight of animals[1] g	
					initial	final
1273	4	crude	10	0	90^{10}	300^{10}
1274	4	»	10	0.1	90^{10}	292^{10}
1275	4	»	10	0.33	90^{10}	304^{10}
1276	4	»	10	1.0	90^{10}	275^{10}
1277	3	»	0	0	90^{10}	273^{10}
1278	3	»	0	0.1	90^{10}	273^{10}
1279	3	»	0	0.33	90^{10}	273^{10}
1280	3	»	0	1.0	90^{10}	292^{10}

[1] The superscripts represent number of chicks in the groups.
[2] The superscripts represent the number of chicks from which the material

from 0 to 0.1 %, but a marked increase occurs in the range between 0.1 and 0.33 % as well as at higher levels.

If the feeding had been continued over a longer period, it is possible that accumulation of cholesterol would have been found at a still lower dietary level.

2. Diets without added fat:

As could be expected (*e. g.* COOK 1936) the increase in cholesterol in liver, spleen, aorta and bile caused by dietary cholesterol was much less than when 10 % peanut oil was present. The increase was insignificant when "vitamin test" casein containing only 0.11 % of fat was used (see fig. 1 A; in this case only liver was examined), and similar results were obtained with bile even when the diet contained the crude casein having a fat content of 1.2 %.

From fig. 1 A and C it can be seen that the initial values (corresponding to 0 and 0.1 % dietary cholesterol) for liver are higher for fat-free diets than for diets with 10 % peanut oil. This agrees with findings of ALFIN-SLATER et al. (1954) who found higher cholesterol values in the liver of rabbits on fat-free diets than in rabbits fed diets with 15 % cottonseed oil.

The observation that in our experiments cholesterol in organs began to increase with dietary cholesterol levels somewhat above 0.1 % agrees with the observations of KATZ and STAMLER (1950), according to which atheromatous lesions could be produced in

3.

liver, spleen, aorta and bile of chicks. Feeding period 4 weeks.

Mg cholesterol per 100 g			
Liver[3]	Spleen[2]	Aorta (dry matter)	Bile[2] (dry matter)
286[10] ± 6	405[10] ± 6	740	530[7] ± 40
303[10] ± 6	410[9] ± 11	820	550[7] ± 70
1,131[10] ± 127	637[10] ± 37	1,450	1,230[8] ± 160
2,536[8] ± 290	995[9] ± 50	3,440	1,510[8] ± 200
384[10] ± 17	407[10] ± 19	840	520[8] ± 50
410[10] ± 25	426[10] ± 12	820	520[7] ± 110
531[10] ± 32	506[9] ± 33	1,130	580[8] ± 70
763[10] ± 94	605[10] ± 44	1,170	660[8] ± 40

in question is analyzed; ± indicates standard error of the mean.

the arteries of chicks fed a plain mash + 5 % cottonseed oil + 0.25 % cholesterol during a 25 to 35 week period from the age of five weeks.

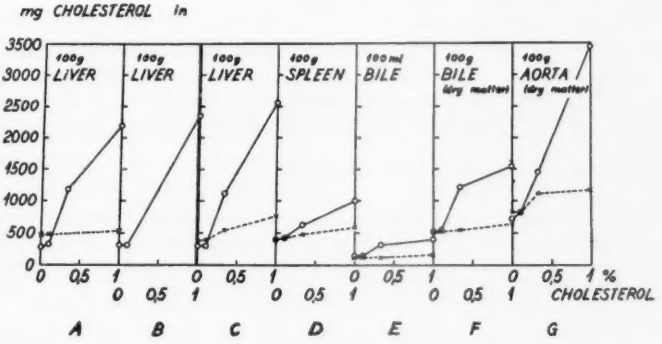

Fig. 1.

Vertical: mg cholesterol.
Horizontal: per cent dietary cholesterol.
Solid lines refer to diets with 10 % peanut oil.
Broken lines refer to diets without added fat.
The casein in fig. 1 A was "vitamin test".
The casein in figs. 1 B—G was crude casein.
The feeding experiments in figs. 1 A and B were carried out simultaneously. Those in figs. 1 C—G were also carried out simultaneously, but later.

Summary.

The dietary cholesterol level above which accumulation could be demonstrated in liver, spleen, aorta and bile was somewhere between 0.1 % and 0.33 % for diets with 10 % peanut oil.

This limit also holds for fat-free diets as far as liver, spleen, and probably also aorta are concerned, whereas in the case of bile only an increase of low significance was found as the dietary cholesterol varied from 0 to 1 %.

The increases found with fat-free diets were in all cases much smaller than those found when the diet contained 10 % peanut oil.

The cholesterol values for liver corresponding to 0 and 0.1 % of dietary cholesterol were significantly higher when the diet was fat-free than when it contained 10 % peanut oil.

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Department of Physiological Chemistry, University of Lund, Sweden.

The Intestinal Absorption of Phospholipids in the Rat.

By

ROLF BLOMSTRAND.

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Since lecithin and triglycerides have structural similarities it is possible that the mechanisms involved in their intestinal absorption might also have points of similarity. Most workers have, however, concerned themselves with the fate of the triglycerides and very little work has been done on the intestinal absorption of phospholipids.

Earlier investigators generally assumed that phospholipids were hydrolyzed in the intestinal lumen and absorbed in their constituent parts. For summaries cf. BELFANTI, CONTARDI and ERCOLI (1936) and ARTOM (1952).

SLOWTZOFF (1906) and FRAENKEL (1911), however, suggested that part of the absorbed lecithin may reach the intestinal lymph unsplit. EICHSTEIN (1924) after feeding lecithin to dogs found as the most pronounced effect an increase in the total fatty acid content of the lymph, but the phospholipid increments were only slight and did not parallel the fluctuations in the total fatty acid content of the lymph. From this he drew the conclusion that lecithin like fatty acids and simple glycerides, is transformed into triglycerides during the transport through the intestinal mucosa.

After prolonged feeding of phospholipids several authors (REWALD 1928, SERIJSKI 1928, and HEINLEIN 1933) have reported increases of the phospholipids of various tissues. These results

have been interpreted as an absorption of intact phospholipid molecules. Recently ARTOM and SWANSON (1948) fed rats with phospholipids obtained from the livers of other rats which had been injected with large doses of ^{32}P. The isotope was also fed as inorganic phosphate or as glycerophosphate to other rats receiving nonlabelled phospholipids in their diet. It was found that the specific activity of the plasma lipids in animals fed labelled phospholipids was much higher than that of the controls and also much higher than that of the liver lipids in the same animal. Since it has been shown that the liver is ordinarily the main source of the plasma phospholipids (cf. HEVESY et. al. 1939, 1940 and FISHLER, ENTENMAN, MONTGOMERY and CHAIKOFF 1943) and since the activity of the phospholipids in these animals was higher in the plasma than in the liver, they concluded that a small part of the phospholipids might be absorbed as intact molecules.

While the present work was still in progress BLOOM, KIYASU, REINHARDT and CHAIKOFF (1954) reported experiments in which they fed liver phospholipids obtained from rats given ^{14}C-palmitic acid to rats provided with lymph fistulas. About 20 per cent of the radioactivity in the lymph was present as phospholipids, but in corresponding experiments with labelled triglycerides only about 3 per cent of the lymph activity was found in the phospholipids. Their interpretation of these results was that a significant portion of fed phospholipids is absorbed without total hydrolysis.

We have previously studied (BLOMSTRAND 1954a) the absorption of phospholipids in lymph fistula rats after feeding phospholipids labelled in the glycerol, fatty acids or phosphate part. The experimental data demonstrated that phospholipids are hydrolyzed to a considerable extent during their absorption in the rat. The results from the experiments with phospholipids and glycerides labelled in the glycerol portion indicated an interconversion of these lipids during absorption. In the present paper these results have been extended. Phospholipids containing oleic acid-1-^{14}C, palmitic acid-1-^{14}C and ^{14}C-glycerol or palmitic acid-1-^{14}C and ^{32}P have been fed to rats with lymph fistulas.

Experimental.

Experiment A. Biosynthesis of phospholipids containg ^{14}C-oleic acid: The thoracic duct of rats weighing about 250 g was cannulated as described by BERGSTRÖM, BLOMSTRAND and BORGSTRÖM (1954). At

least 20 hours after the operation the rats were fed 0.5 ml of methyl oleate-1-^{14}C by stomach tube. Each rat received about $2.5 \cdot 10^6$ c.p.m. The lymph was collected for 24 hours and the lymph fat was extracted with 20 volumes of ethanol : ether 3 : 1 and after evaporation of this extract in vacuo, the residue was extracted with light petroleum : chloroform 2 : 1. The total fat was separated into neutral fat and phospholipids on columns of silicic acid according to BORGSTRÖM (1952 a). The phospholipids were eluted with chloroform : methanol 1 : 1. An aliquot of this solution was used for determination of phosphorus according to Fiske-Subbarow as modified by BRANTE (1949). Another aliquot was used for the determination of fatty acids after hydrolysis of the phospholipids by refluxing for two hours in 10 per cent potassium hydroxide in 50 per cent methanol. After acidification with dilute hydrochloric acid the fatty acids were extracted with light petroleum and directly plated on aluminium planchets in an infinitely thin layer. Phospholipids biosynthesized in this way with labelled oleic acid contained the ^{14}C-activity in the unsaturated fraction after separation of the phospholipid fatty acids into saturated and unsaturated fatty acids according to BERGSTRÖM och PÄÄBO (1954).

Appropriate amounts of olive oil were dissolved in the remainder of the chloroform: methanol solution and the solvent evaporated in vacuo. Rats with the thoracic duct cannulated were then fed 0.5 ml. of olive oil containing about 75 mg of labelled phospholipids with a total activity of $3.0 \cdot 10^4$ c.p.m.

Experiment B. Biosynthesis of phospholipids containing ^{14}C-glycerol and ^{14}C-palmitic acid: Rats weighing about 180 g were fed 0.5 ml of olive oil containing 7 mg of palmitic acid-1-^{14}C by stomach tube. Each rat received about $3.5 \cdot 10^6$ c.p.m.

After 5 hours the rats were sacrificed by cervical fracture and the liver excised and immediately placed in ice-cold buffer solution. The liver (5—6 g) was cut in 5—6 pieces and liver slices prepared free-hand with a thin razor blade. 500—600 mg of tissue was incubated at 37° for 2 hours in 50 ml flasks containing 5 ml of Krebs-Ringer phosphate buffer, pH 7.4 and 0.2 ml of a water solution containing 0.18 mg of glycerol-1-^{14}C with a total activity of $2.4 \cdot 10^5$ c.p.m. The gas phase was air. Following incubation the reactions were stopped by placing the flasks in hot water. The slices were finely dispersed in a Waring blendor, filtered and washed with water several times. The total fat was extracted with 20 volumes of ethanol : ether 3 : 1. The solution was filtered and reduced to a few ml in vacuo. The concentrate was extracted several times with light petroleum and was again concentrated to a few ml under reduced pressure. In order to remove any free radioactive glycerol, a drop of inactive glycerol was added and enough ethanol to give a homogeneous solution. This solution was warmed and the light petroleum and ethanol evaporated in a stream of nitrogen. The mixture of total fat and glycerol was again taken up in light petroleum and washed with water several times. In control experiments under the same experimental conditions as described above in which the tissue was heated immediately, this technique was found suitable to remove

radioactive free glycerol added. The light petroleum solution was dried with anhydrous Na_2SO_4 and again concentrated and the phospholipids were precipitated twice with ice-cold acetone. The phospholipids were finely dissolved in chloroform and brought to volume. Aliquots of this solution were taken for analyses of phosphorus, fatty acids and fatty acid-^{14}C as described above. The acidified solution containing the glycerol portion of the phospholipids was made to volume and aliquots of this solution were plated directly on aluminium planchets. It was found that about 60 per cent of the activity in the phospholipids was contained in the fatty acids and about 40 per cent in the glycerol portion.

An appropriate amount of olive oil was added to the solution of the phospholipids and the chloroform solution was evaporated in vacuo. Lymph fistula rats were then fed 0.5 ml of this solution containing about 100 mg of labelled phospholipids with a total activity of $1.5 \cdot 10^5$ c.p.m.

Experiment C. Biosynthesis of phospholipids containing ^{14}C-palmitic acid and ^{32}P: Three rats weighing about 180 g were fed by stomach tube 0.5 ml of olive oil containing 4 mg of palmitic acid-1-^{14}C with a total activity of $2.0 \cdot 10^6$ c.p.m. At the same time they were given intraperitoneally a large dose of ^{32}P as Na_2HPO_4 (95 μC/rat). After 7 hours they were killed, their livers excised and the total fat extracted with ethanol : ether 3 : 1. After evaporation of this extract in vacuo, the residue was extracted with boiling light petroleum.

This solution was then washed with 1-N hydrochloric acid in order to remove inorganic phosphate and then washed several times with water. The phospholipids were precipitated three times with ice-cold acetone and the precipitate thoroughly washed with acetone. Finally the phospholipids were dissolved in chloroform and the volume made to 50 ml. Aliquots of this solution were taken for analyses of phosphorus, fatty acids and fatty acid-1-^{14}C as described above. The acidified saponified solution was made to volume and aliquots of this solution were plated directly in an infinitely thin layer for determination of ^{32}P. Appropriate amounts of olive oil were dissolved in the chloroform solution and the solvent was evaporated. Lymph fistula rats were then fed 0.5 ml of olive oil containing about 100 mg of labelled phospholipids. Each rat received in this way $2.6 \cdot 10^4$ c.p.m. as ^{14}C-fatty acids and $3.9 \cdot 10^5$ c.p.m. as ^{32}P-phospholipids.

Lymph experiments. Male rats weighing about 250 g were used in this study. The procedure used for cannulating the thoracic duct was that earlier described by BERGSTRÖM, BLOMSTRAND and BORGSTRÖM (1954). The rats were not fasted before insertion of the cannula. Immediately following cannulation the rats had free access to 1 per cent NaCl solution until an adequate lymph flow was obtained. Thereafter they were fed 0.64 per cent NaCl solution with 0.04 per cent KCl and white fat-free bread. At least 20 hours after the operation 0.5 ml of the test mixtures was administered by stomach tube during a light ether anaesthesia and the lymph was collected for the following 24 hours. At this time the rats were killed and the gastrointestinal tract excised. This was combined with the feces collected during the experi-

ment and analyzed for radioactivity after saponification. The amount of radioactivity absorbed was determined from the difference between that administered and that recovered in the gastrointestinal tract and collected feces.

Fractionation of lymph lipids. The lymph fat was extracted with 20 volumes of ethanol : ether 3 : 1 and after the evaporation of this extract in vacuo the residue was extracted with light petroleum : chloroform 2 : 1. The total fat was separated into neutral fat and phospholipids on columns of silicic acid according to BORGSTRÖM (1952 a). The solutions of neutral fat and phospholipids were made to volume and aliquots were taken for radioactivity assay.

The neutral fat was saponified by refluxing with alcoholic potassium hydroxide. The fatty acids were extracted with alkaline 50 per cent ethanol and reextracted with light petroleum. The fatty acids were then directly plated in infinitely thin layer on aluminium planchets and counted. In experiment B the remaining acidified solution containing the glycerol portion of the triglycerides was made to volume. Aliquots of this solution were directly plated on aluminium planchets in infinitely thin layer and the total activity of the fraction calculated.

Samples of phospholipids were refluxed with 10 per cent potassium hydroxide in 50 per cent methanol and the fatty acids extracted with light petroleum after acidification. The aqueous solution containing the glycerol portion of the phospholipids was made to volume. Aliquots were directly plated on aluminium planchets in infinitely thin layer for determination of the ^{14}C-activity in the glycerol part (Experiment B) and the ^{32}P-activity in the phosphate part of the phospholipid molecule (Experiment C).

Results.

Experiment A. From 87 to 92 per cent of the ^{14}C-oleic acid contained in the fed phospholipids was absorbed (Table 1). Of the absorbed activity 59—76 per cent was recovered in the lymph lipids in 24 hours. From 15 to 23 per cent of the activity in the lymph was found in the phospholipids and the remainder in the

Table 1.

Recovery and distribution of activity in lymph lipids after feeding phospholipids containing ^{14}C-oleic acid dissolved in olive oil to rats.

Rat no.	Per cent of fed activity absorbed	Per cent of absorbed activity recovered in lymph lipids	Per cent of lymph activity recovered as	
			Neutral fat	Phospholipids
9	92	59	83	17
10	87	62	77	23
11	90	76	85	15

neutral fat. In figure 1 comparison is made of the partition of the activity in the lymph lipids after feeding phospholipids containing ^{14}C-palmitic acid (BLOMSTRAND 1954a) or ^{14}C-oleic acid and glycerides containing ^{14}C-palmitic acid (BORGSTRÖM 1952 b) or ^{14}C-oleic acid (BERGSTRÖM, BLOMSTRAND and BORGSTRÖM 1954).

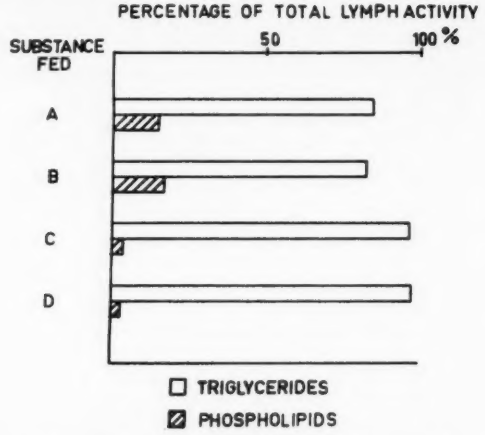

Fig. 1. Partition of ^{14}C-activity in lymph lipids after feeding to rats dissolved in olive oil A. Phospholipids containing ^{14}C-palmitic acid, B. Phospholipids containing ^{14}C-oleic acid, C. Triglycerides containing ^{14}C-palmitic acid or D. Triglycerides containing ^{14}C-oleic acid.

Experiment B. In the experiments with phospholipids labelled in both the fatty acid and the glycerol portion (Table 2) 51—72 per cent of the fed ^{14}C-fatty acid was recovered in the lymph lipids. From 34 to 39 per cent of the ^{14}C-glycerol contained in the fed phospholipids was recovered in the lymph lipids. From 80 to 86 per cent of the activity in the lymph fatty acids was found in the neutral fat and from 14 to 20 per cent in the phospholipids. Of the activity in the lymph glycerol 55—64 per cent was found in the neutral fat and 36—45 per cent in the phospholipids.

Experiment C. After feeding phospholipids containing ^{14}C-fatty acids and ^{32}P (Table 3) 50—81 per cent of the absorbed ^{14}C-fatty acid was recovered in the lymph. Of the absorbed ^{32}P contained in the phospholipids 10—11 per cent was recovered in the lymph phospholipids. The partition of ^{14}C-activity in the lymph lipids was the same as in earlier experiment after feeding phospholipids containing ^{14}C-fatty acids.

Table 2.

Recovery and distribution of activity in lymph lipids after feeding phospholipids labelled with ^{14}C in both the fatty acid and the glycerol portion.

Rat no.	Per cent recovery in lymph of fed		Per cent of activity in fatty acids of lymph found in		Per cent of activity in lymph glycerol found in	
	^{14}C-fatty acid	^{14}C-glycerol	Neutral fat	Phospho-lipids	Neutral fat	Phospho-lipids
17	72	34	86	14	55	45
18	51	39	80	20	64	36

Table 3.

Recovery and distribution of activity in lymph lipids after feeding phospholipids containing ^{14}C-labelled fatty acid and ^{32}P to rats.

Absorption of ^{14}C and ^{32}P; 95 and 96 per cent resp.

Experiment no.	Per cent of absorbed activity recovered in lymph lipids as		Per cent of ^{14}C activity in fatty acids of lymph found in	
	^{14}C	^{32}P	Neutral fat	Phospholipids
41	81	11	88	12
42	50	10	86	14

Discussion.

The results of the experiments with phospholipids containing ^{14}C-oleic acid (Table 1) show that unsaturated fatty acid portion of the molecule is efficiently absorbed mainly via the lymph ducts. There is no difference between a quantitative pattern of absorption of palmitic and oleic acid whether fed as phospholipids or as triglycerides or as free fatty acids (cf. BERGSTRÖM et al. 1954, BLOOM et al. 1954, BLOMSTRAND 1954).

After absorption most of the labelled fatty acids fed in phospholipids were recovered in the lymph lipids (Figure 1). The major part of the activity in the lymph lipids was obtained in the glycerides but both after feeding phospholipids containing labelled palmitic acid and labelled oleic acid a significantly greater amount of activity was obtained in the phospholipids than after feeding these acids as glycerides or as the free acid (cf. BLOOM et al 1954, BLOMSTRAND 1954a). When labelled palmitic acid was fed as the glyceride or as the free acid only about 3—4 per cent of the radio-

activity in the lymph lipids was associated with the phospholipid fraction (BORGSTRÖM 1951, BLOOM et al. 1954, BLOMSTRAND 1954). After feeding labelled oleic acid as triolein or as the free acid only about 2 per cent of the activity in the lymph was recovered in the phospholipids (BERGSTRÖM et al. 1954). In the experiments with phospholipids containing labelled palmitic acid an average of 16 per cent of the lymph activity was obtained in the phospholipids (BLOMSTRAND 1954a) and after feeding phospholipids containing labelled oleic acid an average of 18 per cent of the lymph activity was obtained in the phospholipids. These results are thus in accordance with those of BLOOM et al. (1954) using phospholipids containing labelled palmitic acid. It must be pointed out that the amount of fatty acids which was in the form of phospholipids in the collected lymph was 10—15 per cent of the total fatty acids in the lymph. This is about the same value which has been found during the absorption of free fatty acids or glycerides BORGSTRÖM (1952). In this connection it must also be mentioned that BLOOM et al. (1954) after feeding ^{14}C-labelled tripalmitin together with unlabelled phospholipids obtained the same partition of the activity in the lymph lipids as after feeding labelled tripalmitin alone. The results presented thus indicate that a very extensive hydrolysis of the phospholipids takes place during the absorption process but also that total hydrolysis need not necessarily occur. The finding in this investigation that there was no difference in the partition of the lymph activity after feeding phospholipids containing labelled oleic or palmitic acid is of interest in connection with the results of HANAHAN (1954), which indicated that the lecithin of rat liver only had unsaturated fatty acids in α-position and saturated fatty acids in β-position. It is also interesting that labelled stearic acid is incorporated to a greater extent into the phospholipids of the intestinal mucosa and intestinal lymph than labelled oleic acid (cf. BORGSTRÖM 1952 c and BERGSTRÖM et al., 1954). It is therefore important to investigate the structure of the phospholipids of the intestinal mucosa and the intestinal lymph during fat absorption.

The results of the experiments with phospholipids labelled in the fatty acids and the glycerol portion show that there is a greater recovery in the lymph of the fed phospholipid fatty acids than of the glycerol moiety. Apparently part of the phospholipid fatty acids is set free from the glycerol during the absorption process and the hydrolysis products partly absorbed via different path-

ways. But as a large amount of the ^{14}C-activity in the glycerol portion of the phospholipids fed was obtained in the glycerides of the lymph these must have been synthesized from mono- or diglycerides. This suggestion is supported from experiments with free labelled glycerol by FAVARGER and COLLET (1950) and by BERNHARD et al. (1952) which have shown that this substance is incorporated only to a small extent into the triglycerides or phospholipids of the intestinal mucosa or lymph during fat absorption.

The specific activity of the lymph glyceride glycerol was only about 5 per cent of that of the fed phospholipid glycerol, and therefore only a minor amount of the lymph glyceride glycerol was derived from the fed phospholipid glycerol. But on the other hand the ^{14}C-activity recovered in the lymph glyceride glycerol represented 20—25 per cent of the total activity of the fed phospholipid glycerol. A considerable part of the fed phospholipids had therefore been hydrolyzed during the absorption process; moreover about 60 per cent of the total ^{14}C-labelled glycerol recovered in the lymph had been absorbed as mono- or diglycerides. These findings confirm the results of REISER, BRYSON, CARR and KUIKEN (1952) and BERNHARD, WAGNER and RITZEL (1952) which indicated that a large part of the labelled glycerol of fed triglycerides was absorbed as partial glycerides.

The relative specific activity of the lymph phospholipid glycerol was about 30 per cent. There had thus been a dilution of the absorbed labelled phospholipids with phospholipids from other sources. It has also been shown by REINHARDT, FISHLER and CHAIKOFF (1944) that thoracic duct lymph of dogs contained a significant amount of phospholipids derived from the plasma. Lymph phospholipids are also partly derived from phospholipids synthesized in the small intestine as demonstrated by experiments with labelled fatty acids (BLOOM, CHAIKOFF, REINHARDT and DAUBEN 1951 and BORGSTRÖM 1951). According to these data only about 15 per cent of the fed phospholipids had been absorbed without previous splitting of the glycerol phosphate linkages. The experiments with the ^{32}P-labelled phospholipids also support this suggestion since about 10 per cent of the ^{32}P-phospholipid was recovered in the lymph phospholipids.

The results of the experiments with phospholipids containing ^{14}C-fatty acids and ^{32}P confirm earlier preliminary observations (BLOMSTRAND 1954a) that a certain amount of fed ^{32}P-phospholipid can be recovered in the lymph phospholipids. These results thus

seem to indicate that some phospholipids were absorbed without splitting of the glycerol phosphate bond. Most of the ester linkages between the glycerol and the phosphorus in the phospholipid molecules must, however, be split during the absorption process. These results also support the suggestion that the remaining part of the phospholipid molecule, *i. e.* a mono- or diglyceride is re-utilized for the formation of phospholipids or triglycerides or else degraded further to free fatty acids and glycerol.

Summary.

The intestinal absorption of phospholipids has been studied after feeding rats with lymph fistulas phospholipids containing A. oleic acid-1-^{14}C, B. palmitic acid-1-^{14}C and ^{14}C-glycerol or C. palmitic acid-1-^{14}C and ^{32}P.

The recoveries of the absorbed radioactivity in the thoracic duct lymph was about the same whether ^{14}C-labelled palmitic acid or ^{14}C-labelled oleic acid was incorporated into the phospholipids administered. The major part of the activity in the lymph lipids was associated with the glycerides, but after feeding, phospholipids containing either labelled palmitic acid or labelled oleic acid, a significantly greater amount of activity was obtained in the phospholipids than after feeding these acids as glycerides or as the free acid.

A small amount of the lymph glyceride glycerol is derived from the fed phospholipid glycerol suggesting a synthesis from a partial glyceride.

The relative specific activity of the lymph phospholipid glycerol was about 30 per cent indicating that a significant amount of the fed phospholipids had been absorbed without previous splitting of the glycerol phosphate linkages. The experiments with ^{32}P-labelled phospholipids support this suggestion.

The experimental data thus indicate that a very extensive hydrolysis of the phospholipids takes place during the absorption process, but also that complete hydrolysis does not necessarily occur.

I am indebted to Professor SUNE BERGSTRÖM for placing the labelled compounds at my disposal.

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Department of Physiological Chemistry, University of Lund, Sweden.

On the Intestinal Absorption of Phospholipids in the Rat in the Absence of Bile.

By

ROLF BLOMSTRAND.

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Recently it has been shown by SIPERSTEIN, CHAIKOFF and REINHARDT (1952) and BORGSTRÖM (1953) that in the absence of bile from the small intestine only a small percentage of fed ^{14}C-labelled palmitic acid appears in the intestinal or thoracic duct lymph. As shown from analyses of the feces a rather large amount of the fed labelled fatty acid had nevertheless been absorbed. This finding was in accordance with earlier work by MUNK (1880) and by SHAPIRO, KOSTER, RITTENBERG and SCHOENHEIMER (1936).

Little is known concerning the rôle played by the bile in the absorption of phospholipids. HEINLEIN (1933) stated, after feeding lecithin to dogs with a bile fistula, that phospholipids are not absorbed in the absence of bile. In order to elucidate the absorption of phospholipids in the absence of bile with more sensitive methods, this problem has been investigated with phospholipids containing ^{14}C-fatty acids that were fed to rats with both the thoracic duct and the bile duct cannulated.

Experimental.

Phospholipids containing labelled fatty acids were biosynthesized by feeding rats palmitic acid-1-^{14}C and were isolated from the livers

as described in preceding papers (BLOMSTRAND 1954, 1955). The labelled phospholipids were dissolved in appropriate amounts of olive oil and this mixture was then administered to rats with both the thoracic duct and the bile duct cannulated. Each rat received 0.5 ml olive oil containing about 100 mg of labelled phospholipids with a total activity of $2.0 \cdot 10^8$ c. p. m. and the thoracic duct lymph was collected for the following 24 hours.

The thoracic duct was cannulated as previously described (BERGSTRÖM et al. 1954). The bile duct was cannulated above the entrance of the pancreatic ducts, thus allowing a free flow of pancreatic juice to the intestine. At least 24 hours after the bile fistula had been established the test mixture was administered by a stomach tube under a light ether anaesthesia.

The total fat from the collected lymph was extracted with alcohol: ether 3 : 1 and subjected to chromatography on silicic acid according to BORGSTRÖM (1952). The gastrointestinal tract and the collected feces were analyzed for determination of unabsorbed radioactivity. The chemical methods and the isotopic analyses used were essentially the same as described in previous papers (BERGSTRÖM, BLOMSTRAND and BORGSTRÖM 1954, BLOMSTRAND 1954, 1955).

Results and Discussion.

The results of the experiments are summarized in Table 1. An average of 68 per cent of the fed activity was absorbed. In contrast to the finding of HEINLEIN (1933) in dogs, the results from the present investigation show that in the absence of bile rats are still able to absorb the fatty acids of phospholipids to a considerable extent. In experiments in which phospholipids labelled with ^{14}C-palmitic acid or ^{14}C-oleic acid were fed to rats with only the thoracic duct cannulated the percentage of absorp-

Table 1.

Recovery and distribution of activity in lymph lipids after feeding phospholipids containing ^{14}C-labelled palmitic acid to rats with cannulae in both thoracic duct and bile duct.

Rat no.	Per cent of fed activity absorbed	Per cent of absorbed activity recovered in lymph lipids	Per cent of lymph activity obtained in	
			Neutral fat	Phospholipids
31.....	88.5	17.0	83.2	16.8
32.....	51.5	33.9	87.3	12.7
33.....	59.5	29.1	83.5	16.5
34.....	72.5	24.4	84.0	16.0
	M. 68.0	M. 26.1	M. 84.5	M. 15.5

tion was 82 and 90 per cent respectively (BLOMSTRAND 1954, 1955). There was thus only a minor impairment in the absorption in the absence of bile.

Twenty-six per cent of the absorbed activity was recovered in the lymph lipids. The corresponding figures for the recovery of phospholipids labelled with ^{14}C-palmitic or ^{14}C-oleic acid in rats with intact bile flow to the intestine were 67 and 66 per cent respectively. Like the experiments of SIPERSTEIN et al. (1952) and BORGSTRÖM (1953) these present results suggest that the normal absorption mechanism of transporting the long-chain fatty acids as triglycerides via the lymphatic route operates less efficiently in the absence of bile.

In the present investigation about 85 per cent of the lymph activity was recovered in the neutral fat and about 15 per cent in the phospholipids. The distribution of the activity in the lymph lipids thus showed the same pattern as with a normal bile flow into the intestine (BLOMSTRAND 1954, 1955, BLOOM et al. 1954). Apparently a synthesis of neutral fat from hydrolysis products of the fed phospholipids had taken place during the absorption process. The results thus indicate that bile is not obligatory for hydrolysis of phospholipids even if some aspects of the absorption process are at least quantitatively changed.

Summary.

The intestinal absorption of phospholipids containing ^{14}C-labelled fatty acids has been studied in rats with both the thoracic duct and the bile duct cannulated.

In the absence of bile from the intestine about 68 per cent of the fed activity is absorbed, indicating that bile is not obligate for the absorption of fatty acids fed incorporated into phospholipids. Only about one third of the absorbed labelled fatty acids were transported via the thoracic duct as compared with the normal condition.

The incorporation of the absorbed fatty acids into the neutral fat and the phospholipids of the lymph showed the same distribution whether bile was present or not.

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From the Institute of Physiology and the Institute of Pharmacology, University of Upsala, Sweden.

The Effect of Deuterium Oxide (Heavy Water) on Conduction Velocity in Isolated Frog Nerve.

By

L. GARBY and P. NORDQVIST.[1]

Received 9 February 1955.

Few investigations have been made concerning the influence of deuterium oxide on peripheral nerve function. Using nerve-muscle preparations from frogs, VERZÁR and HAFFTER (1935) found no blocking effect nor any effect on conduction velocity when the nerve was bathed for 15 minutes in a Ringer's solution containing 99.2 per cent deuterium oxide. Using the same technique, HERRMAN (1939) confirmed this finding as far as absence of blocking action was concerned. The test solution used by him contained 99.5 per cent deuterium oxide.

The absence of any effect on the conduction velocity as reported by the above-mentioned authors is not in harmony with modern concepts of conduction in peripheral nerves, unless it is assumed that the nerve membrane is impermeable to deuterium oxide, an assumption which on *a priori* grounds seems unlikely. It was therefore felt that a reinvestigation of the effect of deuterium oxide on conduction velocity in the A-fibres of isolated frog nerve should be undertaken in order to clarify the situation.

Methods.

Isolated sciatic nerves from winter frogs (Rana temporaria) were used exclusively. The nerves were placed in Ringer's solution for about half an hour before mounting on the electrodes. In some of the experiments, the connective tissue sheath of the nerve was removed.

[1] Present address: Medical clinic I, Sahlgren's hospital, Gothenburg.

The Ringer's solutions used were of two kinds. Each solution contained 0.647 % NaCl, 0.0186 % KCl, 0.0200 % $NaHCO_3$ and 0.0120 % $CaCl_2$, but one was made up with distilled water and the other with a water containing 99.97 per cent deuterium oxide ("Norsk Hydro"). These solutions will in the following be called H_2O-Ringer and D_2O-Ringer respectively. The pH of the solutions varied between 6.8 and 7.0. In each experiment, however, test and control solutions had the same pH within 0.1 unit.

The nerves were placed in contact with the test solutions in three different ways. In the first series of experiments, the nerve passed through a cylindrical bath, 2 cm in diameter and 3.5 ml in volume, containing the solutions. Stimulating electrodes were placed on one side of the bath and recording electrodes on the other. These experiments were carried out in an atmosphere of pure oxygen. In the second series of experiments, the whole nerve, mounted on the electrodes, was immersed in the test solution. The fluid volume in this case was 20 ml. During the actual recording, the electrodes with the nerve were lifted out of the bath into air. In the third series of experiments the nerve was left immersed in the solution (15 ml) also during the recording. This could be made by allowing the distal end of the nerve to pass through a hole, 11 mm long and 0.5 mm in diameter, in a Perspex block and the proximal end through a hole of the same length and with a diameter of 0.8 mm. Stimulation and recording was made in these regions of the nerve by means of silver electrodes inserted in the blocks 7 mm apart. The distance between the stimulating cathode and the grid lead varied between 50—76 mm. Conventional methods for oscillographic recording were used. Supramaximal stimulation was given with a frequency of 1 per second, but during photographic recording a frequency of 10 per second was used. All experiments were photographed using an exposure time of 1 second. The apparatus used was described by NORDQVIST (1954).

The experiments were carried out at room temperature (19.5°—22.0° C). The variation in temperature during each experiment was less than 1.0° C.

In most experiments the nerve was first placed in H_2O-Ringer and two recordings were made 5 minutes apart. After that, the nerve was immersed in D_2O-Ringer and recordings were taken every 5th minute for some time. Thereafter, the nerve was again immersed in H_2O-Ringer and recordings taken every 5th minute. In some experiments, the conduction velocity was recorded each minute during the first five minutes after changing the solution and in one experiment every 10th second of the first minute after changing to D_2O-Ringer, which took 25 seconds.

Results.

Twenty-three experiments were carried out. In all these experiments a decrease in conduction velocity was noted during treatment with D_2O-Ringer. The effect was always reversible. The results

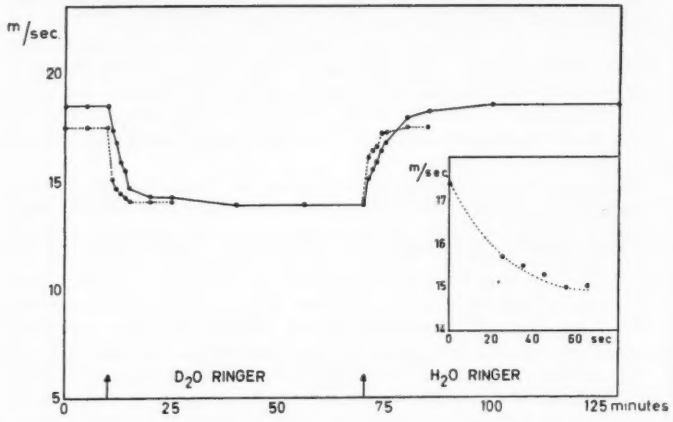

Fig. 1. This figure is a graphical representation of the experiments 8 (full line) and 9 (dotted line). Ordinate: conduction velocity. Abscissa: time. The arrows ndicate immersion in D_2O-Ringer and H_2O-Ringer respectively. The insert shows the time course of conduction velocity during the first minute after immersion in D_2O-Ringer of the nerve in experiment 9.

of the second and the third series of experiments, *i. e.* with the whole nerve in contact with the test solution, are shown in table 1. Figure 1 shows the result from experiments no. 8 and no. 9, and a graph of the recordings taken every 10th second of the first minute after changing to D_2O-Ringer in experiment no. 9 is shown as an insert.

The table and the figure show that the decrease in conduction velocity is close to maximal already after 5 minutes and that normalisation during reimmersion in H_2O-Ringer takes place at about the same rate. The magnitude and the rate of decrease is approximately the same in intact nerves and those without sheath. In the experiments where recordings were made with nerves immersed in 15 ml of liquid, the effect seems to be slightly larger than the corresponding effect obtained when the recording was made in air, with only a thin film of liquid adhering to the nerve.

The effect of deuterium oxide on the magnitude of the action potential is shown in figure 2. No measurable effect is seen after 2.5 hours of immersion in D_2O-Ringer. The small biphasic change

Table 1.

Solution	Time after immersion in new solution. Minutes	Per cent decrease from initial velocity in H_2O-Ringer									
		Recording in air						Recording in liquid			
		Intact nerve				Unsheathed		Intact nerve		Unsheathed	
		1	2	3	4	5	6	7	8	9	10
H_2O-Ringer	5	0	0	0	0	0	0	0	0	0	0
	10	0	0	0	0	0	0	0	0	0	0
D_2O-Ringer	1	0				3	5	9	6	14	8
	2	0				8	10	11	10	16	16
	3	3				10	13	15	14	17	19
	4	5				10	13	19	16	18	21
	5	9	9	25	10	10	14	21	20	20	21
	10	15	19	26	18	10	14	24	23	20	21
	15	15	19	26	18	10	15	26	23	20	21
	20							26			
	25							26			
	30							26	25		
	60							26	25		
H_2O-Ringer	1	13				3	10	19	19	8	16
	2	13				3	5	17	16	6	12
	3	10				0	3	17	14	5	8
	4	8				0	0	11	9	2	5
	5	8	10	11	10	0	0	11	3	2	2
	10	0	5	7	0	0	0	9	2	0	0
	15	0	5	0	0	0	0	6		0	0
	20							4			0
	25							0			
	30							0	0		
	60							0	0		
	Initial velocity in H_2O-Ringer m/sec.	20	20	18	16	20	20	19	18	17	17

of the action potential after this period, indicated in the centre of the picture, is to be expected since the injured part of the nerve was not immersed in KCl.

Discussion.

Working with isolated single nerve fibres from *Carcinus maenas*, Hodgkin (1939) and Katz (1947) showed that an increase in the resistance of the surrounding medium causes a decrease in the velocity of propagation. These experiments support strongly

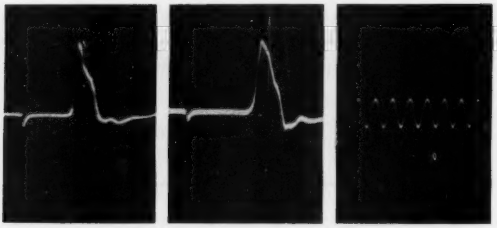

Fig. 2. Photographic recording of the action potential a) before and b) after immersion for 2.5 hours in D_2O-Ringer. c) 1,000 periods/sec.

the local circuit theory of nervous transmission, the essential points of which are assumed to be valid also for myelinated fibres. The following discussion is based on this theory.

Impulse transmission in peripheral nerves depends on rate processes such as chemical reactions and transport of ions and molecules. The effect of deuterium oxide on such processes is generally assumed to be an increase in the energy of activation of the process. Thus, the ratio between the specific conductance of an approximately 100 mM NaCl solution in H_2O and D_2O is 1.23 at 18° C (TRONSTAD and STOKLAND 1937). This is in accordance with the generally accepted view that the velocity of ion transport processes is dependent on the viscosity of the medium, the ratio of the viscosity between D_2O and H_2O being 1.25 at 20° C. The effect of increased viscosity on ion transport velocity is also reflected in the temperature coefficient for diffusion, which is about the same as that for viscous flow. The replacement of H_2O with D_2O in the present experiments is, from the point of view of ion transport in a watery medium, equivalent to a decrease in temperature of about 10°C. Now, the temperature coefficient, Q_{10}, of conduction velocity in isolated frog nerve has been estimated to be 1.78 (LUCAS 1908). The present experiments however show a decrease in conduction velocity of only about 20 per cent after equilibration with D_2O-Ringer. This indicates that there are, in addition to ionic migration, other processes, the rates of which are affected by temperature changes rather than by changes in viscosity in the water phase. A reasonable guess would be that these processes are chemical reactions, most of which have temperature coefficients between 2 and 4. However, it must be remembered that comparatively little is known about the activation energies for ion migration across barriers

which contain little or no water. Such processes probably have temperature coefficients larger than 1.5 (cf. DAVSON and DANIELLI 1952), and they might well be relatively unaffected by the introduction of deuterium oxide.

The site of action of the deuterium oxide cannot be evaluated at present because no complete theory exists that can relate propagation velocity in myelinated fibres with those parameters which are supposed to be changed in the present experiments (cf. RUSHTON 1952).[1] However, an effect on the membrane and/or the axoplasm is most probable since the effect on outside electrical resistance is negligible on account of the very large outside volumes in experiments 7—10.

The reason why VERZÁR and HAFFTER (1935) did not find any effect probably depends upon the lower sensitivity of their recording system.

The experiments furthermore provide data concerning the permeability of the myelin sheath to water. Let us assume that at least part of the decrease of conduction velocity is due to the entrance of deuterium oxide into the axoplasm. Since conduction velocity becomes constant within 5 minutes in an unsheathed nerve, virtually complete exchange equilibrium for water between the axoplasm and the external medium must be reached within that time. An approximate estimation of the resistance of the myelin sheath to water diffusion may then be carried out as follows: If it is assumed that the myelin sheath is impermeable to water, the axoplasm will exchange its water only by way of the nodal membrane. If the axoplasm immediately under this membrane contains D_2O at a concentration c_e, the concentration of D_2O, c, at any point and time in the axoplasm between the nodes will be given by the following solution of Fick's law for one-dimensional diffusion:

$$c = c_o \left(1 - \frac{2}{\sqrt{\pi}} \int_0^y e^{-y^2} \cdot dy \right)$$

where $y = \frac{x}{2\sqrt{D \cdot t}}$, x is the distance from the node, D is the integral binary diffusion coefficient for the system D_2O—H_2O and t is the time. The use of the "free" diffusion coefficient for D_2O

[1] We wish to express our thanks to Dr. W. A. H. RUSHTON, F. R. S., for discouraging from proceeding with any interpretations in this respect.

in the present case is justified by the fact that HODGKIN and KEYNES (1953) found the mobility of potassium ions in the axoplasm of *Sepia* to be very close to that in water.

For $x = 1$ mm (half the internodal length) and $t = 5$ minutes, c is found to be only 38 per cent of c_0, the latter being assumed constant during this time. It is thus seen that water penetration across the myelin sheath makes up for at least the lacking 62 per cent at the central portion of the internodal region, even if we assume that, from the very start of the experiment, we have a constant composition of 100 per cent D_2O immediately beneath the nodal membrane. These calculations do not say anything about the actual permeability of the myelin sheath in terms of the diffusion coefficient of water in it, but they certainly seem to indicate that *the nerve can exchange its water also across the myelin sheath.*

Summary.

The effect of Ringer's solution containing 99.9 % deuterium oxide on the conduction velocity of the A-fibres in isolated frog nerves was investigated.

A decrease in conduction velocity of about 20 % was noted, the full effect being reached in about 5 minutes in unsheathed nerves and in about twice that time in intact nerves.

A rapid exchange of water between the nerve and its environments could be inferred from the rate of decrease in conduction velocity. A considerable part of the exchange must take place across the myelin sheath.

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From the Department of Physiology, Karolinska Institutet, and the Endocrine Division of the Department of Internal Medicine, Serafimerlasarettet, Stockholm.

The Urinary Excretion of Noradrenaline and Adrenaline in Healthy Subjects During Recumbency and Standing.

By

U. S. VON EULER, R. LUFT and T. SUNDIN.

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Evidence has been produced lately to show that a functional differentiation exists between the physiological actions of adrenaline and noradrenaline. Thus adrenaline is selectively released from the suprarenal medulla and excreted in the urine during hypoglycemia (DUNÉR 1953, EULER and LUFT 1952), while noradrenaline is predominantly released during the general pressor reflexes elicited by carotid occlusion (KAINDL and EULER 1951).

It therefore seemed of interest to determine whether or not the reflex vasomotor activity, known to operate during normal standing, is accompanied by an increased excretion of noradrenaline in urine as a sign of increased production.

Material and Methods.

The investigation was performed in 15 healthy students 21—28 years of age. They were kept in bed in the laboratory during the night before the test. They emptied the bladder at 5 a. m. (still lying down) and were then allowed to drink a glass of tap water. They were then allowed to sleep until 8 a. m. when the bladder was emptied again. This 3-hour urine specimen was used for the determination of the excretion of catechol amines during recumbent position. At 8 a. m. the subject was put on a tilting table which was kept at an angle of 75° and maintained in this position for 3—4 hours, while pulse rate and

blood pressure were registered at regular intervals. Urine was collected at 9, 10 and 11 o'clock, and in 7 out of 15 experiments at 12 o'clock.

The free catechol amines of the urine were extracted according to EULER and ORWÉN (1955), and subjected to quantitative assay of adrenaline and nordrenaline. As test objects the cat's blood pressure and the hen's rectal caecum were used.

In order to exclude effects possibly due to diurnal variations in the excretion of catechol amines 10 healthy subjects were studied while remaining in the supine position during the whole time of the experiment, *i. e.*, from 5 to 12 a. m. The bladder was emptied at 5, 8, 9, 10, 11 and 12 o'clock.

In a number of experiments a light meal was given immediately before the subjects were placed on the tilting table. This was done in order to study whether the fasting to 12 o'clock influenced the excretion of adrenaline and noradrenaline.

Results.

The excretion of adrenaline between 5 and 8 a. m. with the 15 subjects in the supine position was 3.9 ± 0.9 mμg and of noradrenaline 10.9 ± 1.5 mμg per minute. These figures were very similar to those observed in the group which remained in the supine position during the entire time. Between 5 and 8 a. m. the excretion of adrenaline in this group was 3.8 ± 1.9 mμg per minute and of noradrenaline 8.5 ± 1.9 mμg per minute.

During 75° inclination for 3—4 hours the output of adrenaline increased to 12.4 ± 1.8 mμg and that of noradrenaline to 30.9 ± 4.1 mμg per minute (Table I). Figure 1 shows these changes in one of the subjects.

Table I.

Difference in urinary output of catechol amines before and during tilting.

Position	No. of subjects	Mean excretion		Time o'clock
		l-adrenaline mμg/min.	l-noradrenaline mμg/min.	
Recumbent (before tilting) ...	15	3.9 ± 0.9	10.9 ± 1.5	05.00 — 08.00
Standing (tilted 75°) ...	15	12.4 ± 1.8	$30.9 — 4.1$	08.00 — 11.00 (12.00)
Diff.		8.5 ± 2.0 $p = < 0.001$	20.0 ± 4.4 $p = < 0.001$	

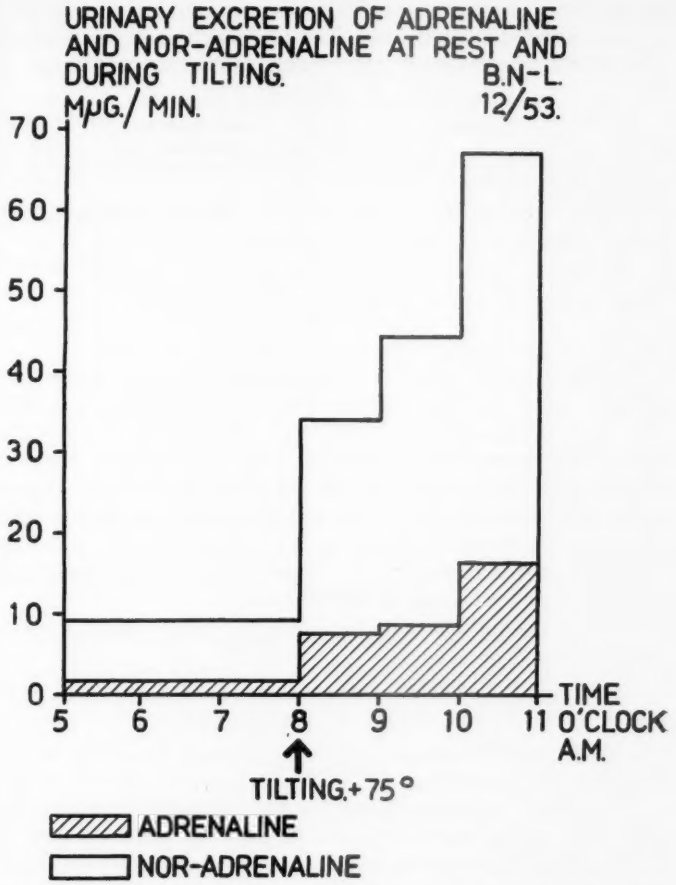

Fig. 1. Effect of tilting + 75° on the urinary excretion of adrenaline and noradrenaline in subject B. N. Ordinate mμg/min.; Abscissa Time, hours.

When these figures are compared with those obtained in the group which remained in the supine position very marked differences are observed. In this group the excretion between 8 and 12 a. m. was only 4.7 ± 0.7 and 12.3 ± 3.1 mμg per minute respectively indicating that the shift from supine to erect position caused a considerable extra output of catechol amines (Table II).

Table II.

Urinary output of catechol amines during 7 hours in recumbent position.

Position	No. of subjects	Mean excretion		Time o'clock
		l-adrenaline mμg/min.	l-noradrenaline mμg/min.	
Lying in bed (asleep)	10	3.8 ± 1.2	8.5 ± 1.9	05.00 — 08.00
Lying in bed (awake)	10	4.7 ± 0.7	12.3 ± 3.1	08.00 — 12.00
Diff.		0.9 ± 1.4	3.8 ± 3.6	

During the first minutes after assuming the erect posture the systolic blood pressure of the subjects decreased by 10—15 mm Hg, in no case, however, reaching a value below 100 mm Hg. The systolic pressure then returned to the initial value where it stayed for the remaining part of the period of standing. The diastolic pressure remained unchanged in most of the tests; a slight increase was observed in only a few subjects during standing. The pulse rate increased by 15—25 per minute during the change of position and remained at this increased level.

None of the subjects noted any marked inconveniences during standing for 3 or 4 hours.

In the group which remained in bed the pulse rate and blood pressure remained unchanged during the test.

Discussion.

The urinary excretion figures for adrenaline and noradrenaline during the control period with the subjects in recumbent position agree well with the values obtained during night hours by EULER and LUNDBERG (1954) and EULER, HELLNER-BJÖRKMAN and ORWÉN (1955).

The considerably increased excretion figures of noradrenaline during standing as compared with those obtained during the same hours during recumbent position indicates that the effect is elicited by the change in position. Since the noradrenaline excretion in urine gives a relative measure of the release in the body, the results strongly suggest that the increased excretion is induced by a stimulation of the noradrenaline-producing vasomotor nerves by the orthostatic fall of the blood pressure, acting over

the blood pressure homeostatic reflex mechanisms of the carotid sinus and aorta. This interpretation is also supported by the findings of LUFT and EULER (1953) that the noradrenaline excretion is very low in cases of postural hypotension, where blood pressure cannot be maintained.

The moderate increase in the excretion of adrenaline during standing might be due to the muscular stress or inconvenience accompanying standing for 3 to 4 hours. When the subjects were kept in a recumbent position during the whole test no such increase appeared. The fasting during the test did not cause this increase in urinary adrenaline; it also appeared when the subjects were given a meal before standing up.

Our preliminary data are in agreement with the supposition that the blood pressure homeostasis in standing is maintained by a reflex stimulation inducing a noradrenaline secretion from the vasomotor nerve endings and possibly the adrenal medulla.

The increase in noradrenaline excretion during erect posture may therefore serve as a test for the degree of orthostatic stress imposed upon the organism in this condition.

Summary.

Healthy persons placed on a tilting table at an angle of 75 degrees for 3 or 4 hours show a considerably increased urinary output of noradrenaline in comparison with the corresponding excretion in recumbent position.

The increased production of noradrenaline is interpreted as the result of reflex activation of the vasomotor system induced by the orthostatic fall of the systolic blood pressure.

The excretion of adrenaline in urine during the tilting test showed a slight to moderate increase probably depending on various stress factors (muscular activity, uncomfortable position) during standing.

In none of the 15 tested healthy persons on the tilting table (75°) showing increased noradrenaline excretion, did syncope occur.

Acknowledgements.

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From the Medical Clinic B, University of Liége, Belgium.

The Action of Chronic Denervation and of the Use of a Ganglioplegic or a Sympatholytic Agent on the Baresthetic Device of the Renal Arteries.

By

L. BRULL, D. LOUIS-BAR and H. LYBECK.

Received 11 February 1955.

From the time one of us (BRULL 1931) devised a convenient method for transplanting kidneys in the neck, with a simple method for measurement of blood flow and oxygen consumption, we were struck by the fact that, whereas arterial pressures below let us say 100 mm Hg reduce the blood flow through the kidneys, variations within or slightly above normal limits usually fail to influence the flow (DOR M. and BRULL L. 1940).

This was not a new finding. UNNA (1935) reported that clamping of the carotid in the dog, while increasing the systemic pressure, did not alter the renal blood flow measured with the thermostromuhr.

HARTMANN, ØRSKOV and REIN (1937) confirmed this fact, and added that dividing the renal nerves on one side did not modify such response. These authors speak of a »mechanism which maintains a constant blood flow in the kidney".

But it was not until we had at our disposal the mechanical heart with coagulable blood (BRULL 1950) that we were able to submit the kidney, either in situ or transplanted in the neck, and still perfused with normal blood from the unoperated animal, to any given pressure up to maximum level of resistance of the renal capsule ($\pm$ 330 mm Hg), and that we could investigate the problem of the renal vascular tone under proper conditions.

Our experiments have gone through several stages; we published the results of the first three which consisted in the study of the behaviour of the blood flow through the kidneys at different pressure levels when

1. transplanted in the neck, in acute experiments (acute denervation (BRULL and LOUIS-BAR 1953);
2. perfused in situ, with preservation of the nerves (BRULL and LOUIS-BAR 1953);
3. perfused with blood from hypophysectomised dogs (BRULL and LOUIS-BAR 1953).

The results of those first three stages of our research were, mainly:

1. that in acute transplanted kidneys, the venous output is very independent of the arterial pressure, a positive response being obtained in only one case out of three, and that, under such conditions (chloralose anaesthesia) a progressive vasoconstriction develops in the renal vessels (also in one case out of three);

2. that innervated kidneys do practically not respond, even to perfusion pressures over double the normal figures, by a change in venous output;

3. that possibly, as VERNEY pointed out in 1926, the pituitary gland contributes to the blood a vasomotor substance which might increase the vascular tone of the kidney.

Yet, when we began using our mechanical heart to study our problem, the dominant hypothesis, to us, seemed that the blood might convey to the kidney one or several substances (hormones?) which regulate the blood flow, while further research, without eliminating this hypothesis at all, stressed more and more the importance of the renal nerves.

On the other hand, after the evidence in favour of the hypothesis that the kidney possesses *a peculiar baro-aesthetic device* regulating its own blood flow, had been confirmed, we thought we would suspend our work on kidneys for a while, so as to find out whether a similar regulating mechanism exists in other parts of the body.

Experiments on submaxillary glands in the dog (BRULL and LOUIS-BAR 1953), and on the hind limbs of the same animal (BRULL and LOUIS-BAR 1953) showed that even when innervated, there seems to be no such mechanism in those parts of the body, and that they regularly respond to increasing levels of perfusion pressure by an increase of blood flow of the same magnitude.

We thought it would be of utmost interest to thoroughly investigate the influence of the renal nerves. *The object of the present paper is to describe and discuss the results of experiments on dogs in which pharmacologically denervated kidneys on one hand, and chronic denervated kidneys on the other, were submitted, from eighteen hours up to one month after aseptic denervation, to perfusion with our mechanical heart in order to measure their vascular tone, and to confirm our hypothesis as to existence and nature of a baro-aesthetic mechanism in the kidney vessels.*

I. Pharmacological Denervation.

We tried to eliminate the interference of the renal nerves by an i.v. injection of a ganglioplegic drug, Pendiomide * or by Scurocaine, immediately after chloralose, and before the kidneys were transplanted in the neck and submitted, through our mechanical heart, to step-wise increased blood pressures.

Table I condenses the results of seven of these experiments. In each case, we give only three or more levels of perfusion pressure, the corresponding renal venous flows (in ml per g of fresh kidney), and the time; zero time is time at which the drug was given.

Table 1.

No. of experiment	Time in min.	Blood pressure (mm Hg)	Venous flow	Remarks
253	0			120 mg chloralose and 1.2 mg Pendiomide (per kg) to kidney dog
	60			50.0 mg Scurocaine into aorta above kidneys
	112	190	4.7	
	132	300	5.4	magnitude of response 25 %
	148	100	3.0	
	158	120	3.4	
	170	190	4.2	
	178	280	4.9	
	185	300	5.0	response 35 %
254	0			120 mg chloralose and 1.2 mg Pendiomide per kg i.v.
	160	90	1.8	no Scurocaine
	166	140	2.4	
	187	305	2.8	response 23 %
	192	240	2.1	
	198	135	1.9	
	214	315	2.9	response 30 %
	216	180	1.5	

[1] kindly put at our disposal by the Ciba Co.

No. of experiment	Time in min.	Blood pressure (mm Hg)	Venous flow	Remarks
258	0			120 mg chloralose and 1.2 Pendiomide per kg i.v.
	120			100 mg Scurocaine into aorta above kidneys
	180	140	6,5	
	191	220	7.5	
	195	160	7.0	
	197	290	11.4	response 67 %
	202	150	6.0	
261	0			120 mg chloralose and 1.2 mg Pendiomide per kg i. v.
	120			400 mg Scurocaine i.v. into perfusor
	136	80	2.0	
	146	110	2.4	
	151	195	3.1	response 42 %
255	0			100 mg Scurocaine into aorta above kidneys
	40	170	3.7	(60 minutes after chloralose)
	61	95	2.0	
	71	120	2.8	
	84	160	3.5	
	94	210	3.8	
	97	270	4.0	
	109	200	3.8	
	129	300	4.6	response 35 %
256	140			80 mg Scurocaine into aorta above kidneys
	153	100	4.7	
	162	160	5.2	
	170	225	5.2	
	175	310	5.2	magnitude of response 5 % (insufficient dose of Scurocaine?)
	177	100	2.7	60 mg Pendiomide into perfusor (19 kg) pressure of the animal falls from 120 down to 45 mm.
	195	160	2.7	
	199	220	2.6	
	201	270	2.0	Pendiomide after denervation does not prevent vasoconstriction
268	140	155	3.2	140 min. after chloralose. No Pendiomide or local anaesthetic; kidneys were denervated 8 days before
	145	205	3.6	
	152	265	4.2	magnitude of response 14 %
	160	180	2.8	
	165			25 mg Pendiomide i.v. into perfusor weighing 22 kg (small dose)
	167	80	2.5	
	172	155	3.0	
	174	320	3.2	response 9 % (slight progressive vasoconstriction
	178			caffeine benzoate 1.2 g i.v. into perfusor
	181	140	4.4	
	183	300	6.2	response 28 % (vasodilatation)
	191	140	3.4	

Table II condenses the results of eleven experiments in which the kidneys were denervated for periods of one day up to one month before they were transplanted into the neck of another dog (under chloralose), and submitted to step-wise rising perfusion pressures, while their blood flow was measured.

Table 2.

Chronic denervated kidneys.

No. of experiment	Time in min.	Blood pressure (mm Hg)	Venous flow	Remarks
271	0			chloralose to the kidney dog
	90	140	5.6	
one day	94	175	7.0	
after	99	240	7.8	
denerv.	102	300	8.3	response 69 %
	107			10 mg Regitin i.v. to perfusor (22.3 kg), produces no drop of pressure
	117	80	3.6	
	118	160	5.9	
	120	220	7.1	response between 160 and 220 mm 40 %
	123	310	8.0	
	128			1 g caffeine benzoate i. v.
	132	60	3.1	
	140	90	5.0	
273	90	165	1.8	
2 days	97	300	3.4	response 100 %
after	100	155	1.4	
denerv.	101	220	3.2	response 300 %
	104			10 mg Regitin i.v. to perfusor (27.2 kg) produces a drop of pressure from 105 to 60 mm
	109	150	2.4	
	115	320	4.8	response 90 %
276	138	150	3.5	
	146	205	5.3	
	147	320	7.5	response 100 %
3 days	150			20 mg Regitin into aorta above kidneys; drop
after	157	150	3.7	of pressure from 175 to 80 mm
denerv.	159	310	8.0	
	165	150	4.5	
	167	320	8.0	response 190 %
263	96	110	2.4	
7 days	105	125	3.0	
after	109	180	4.5	response 124 %
denerv.	128	280	5.3	
280b	115	120	3.2	
	118	200	3.6	
8 days	112	200	3.6	response 4 %
after	114			20 mg Regitin i.v. into aorta above kidneys
denerv.				drop of pressure in perfusor from 130 to 80 mm

No. of experiment	Time in min.	Blood pressure (mm Hg)	Venous flow	Remarks
	120	120	3.2	
	130	120	2.9	
	133	300	4.2	response 26 %
	135	135	3.3	
	139	320	5.7	response 51 %
268b	140	155	3.2	
	145	205	3.6	
8 days	152	265	4.2	response 43 %
after	160	180	2.8	
denerv.				
	165			25 mg Pendiomide i. v. to perfusor (22 kg)
	167	80	2.5	(no drop in pressure)
	172	155	3.0	
	174	320	3.2	
	178			caffeine benzoate 1.2 g into perfusor
	181	140	4.4	
	183	300	6.2	response 33 %
	191	140	3.4	
286	120	115	3.0	
14 days	142	170	4.2	
after	144	270	6.7	response 90 %
denerv.				
266	97	145	5.0	
16 days	110	290	6.0	response 20 %
after	116	155	4.5	
denerv.	119	290	6.0	
268a	91	140	5.6	
1 day	100	190	6.6	
after	103	250	8.1	response 55 %
denerv.				
287	110	150	2.5	
24 days	114	300	3.7	response 48 %
after	117	150	2.7	
denerv.				
	120	305	3.7	
	122			20 mg Regitin into aorta above kidneys (drop of pressure in perfusor of 22.3 kg, from 140 to 80 mm)
	127	150	3.9	
	128	210	4.1	
	129	310	4.8	
	134	150	4.6	
	136	310	5.3	response 14 %
288	70	130	2.2	
30 days	73	180	2.3	
after	75	300	2.5	
denerv.	80	120	2.5	
	82	310	4.2	response 43 %

Discussion.

In the five experiments in which Pendiomide was given as a ganglioplegic before the kidneys were transplanted, and eventually Scurocaine (in four cases) also was injected into the artery leading the blood to the transplanted kidneys, there is a dilatation of the renal vessels when perfusion pressure is increased by 50 or 100 per cent. The percentage of response, as given in the tables, means the ratio of increase in blood flow to the increase in pressure. This response varies from 30 up to 67 %, and is of the same magnitude as the response obtained in acute transplantations where no drug is given. The only difference is that in the last there are positive responses only in one case out of three, while here all five answers are positive.

How do chronic denervated kidneys behave? In ten cases out of eleven, there is a positive response, varying from 14 up to 300 per cent. In one case, the response is only 4 %, which may be considered as nil, or within the error limits of measurement.

There is no doubt that we have taken another step forward in eliminating the mechanism of homeostasis of the renal blood flow. From the innervated kidneys which do not respond, through the acute denervated kidneys which respond in one case out of three, we have reached a stage, either by pharmacological denervation or by chronic denervation, at which we have practically 100 % positive answers. Yet the variability of such answers leads us to believe that by eliminating sympathetic innervation as much as possible, or at least by disconnecting it, we have in some cases reduced the renal arteries to mere elastic tubes.

Still, our success is variable and incomplete. It is clear to us that further research is necessary, and we may have to revert to our first research hypothesis, namely that hormones interfere. Are they postpituitary or adrenaline-like substances carried by the blood, or similar substances liberated in the renal arterial wall while it is stretched? These are the subjects of our present speculations and experimental attempts.

While this paper was being written, Professor VERNEY informed one of us (BRULL) of recent experimental results, now in the press (Philos. Trans. 1955); they are of the highest interest, as they were obtained from unanaesthetised dogs. They show that occlusion of both carotids (with innervated sinus) raising the systemic

pressure up to 195 mm is not followed by a noticeable increase of the renal blood flow, as measured with PAH under puncture of the renal vein. VERNEY thinks of an intrinsically developed increase in the resistance of the proglomerular vessels. One might perhaps suggest that the resistance is rather at the outflow of the glomeruli, if one wishes to understand what clearly appears from a paper published in 1953, where we showed that doubling the perfusion pressure, while eventually not altering the blood flow, may considerably increase the urine flow.

Summary.

Proceeding with our research on the mechanism of the baresthetic device regulating the constancy of the renal blood flow, by submitting kidneys transplanted in the neck to step-wise raised perfusion pressures, by means of the mechanical heart with coagulable blood, we found that chronic denervation, or the use of a ganglioplegic (Pendiomide) or of a sympatholytic (Scurocaine), eliminates totally or partially vasoconstriction of the renal arteries which occurs in response to a rise of pressure and prevents variations of the blood flow.

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The Nutrition Research Institute, University of Oslo, Norway.

The Effect of Dietary Fat on the Intestinal Cholesterol Absorption and on the Cholesterol Metabolism in the Liver of Rats.[1]

Cholesterol Studies III.[2]

By

ALEXANDER PIHL.[3]

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In recent years the main pathways of the biochemical synthesis and degradation of cholesterol have been fairly well outlined. However, various fundamental physiological and nutritional aspects of cholesterol metabolism remain obscure. The important interrelationship between fat and cholesterol metabolism, which is the subject of the present paper, is thus poorly understood. This applies to the rôle of dietary fat in the absorption, as well as in the intermediary metabolism of cholesterol.

It has been generally assumed until recently that cholesterol is absorbed in rats only in the presence of dietary fat. This view was mainly based on the work by COOK (1936, 1937). He found, in balance experiments, that cholesterol given in a fat free diet was quantitatively recovered in the feces. Furthermore, in prolonged feeding experiments, when fat was absent from the diet, cholesterol did not accumulate in the liver. On the other hand, BOLLMAN and FLOCK (1951) found definite evidence that dietary

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[2] Cholesterol Studies I and II were published in Scandinavian Journal of Clinical and Laboratory Investigation.

[3] Fellow of the Norwegian Cancer Society.

Present address: Norsk Hydro's Institute for Cancer Research, The Norwegian Radium Hospital, Oslo.

fat is not necessary for cholesterol absorption in experiments where the absorption was gauged by measurements of the cholesterol content of intestinal and hepatic lymph. Further evidence in support of this view was later obtained in balance experiments (KIM and IVY 1952).

The quantitative effect of dietary fat on cholesterol absorption has been studied systematically only by few investigators. COOK (1937) studied the effect of varying levels of fat and found no difference in the absorption when rats were given 2 per cent cholesterol with 15, 20 and 30 per cent arachis oil respectively. LOIZIDES (1938), on the other hand, observed a progressive and very marked increase in the deposition of cholesterol in the liver of rats given 2 per cent cholesterol with 11, 21, 31, and 41 per cent fat. The results were attributed to an effect on cholesterol absorption, although direct measurements of the absorption were not carried out. KIM and IVY (1952) recently observed in rats that fat did only increase cholesterol absorption when it was present in the diet in large amounts compared to cholesterol. Thus no stimulating effect of fat was found when the proportion of fat to cholesterol was 4 : 1 or 8 : 1. In these test periods the absorption was no greater than in the control groups given the cholesterol in the absence of fat. When the proportion of fat to cholesterol was raised to 24 : 1, a definite effect of fat was found. Intermediate proportions were not tested.

It is apparent from the preceding that further data on the effect of dietary fat on cholesterol absorption are needed. In the present communication such data, obtained in balance experiments, are offered. The balance experiments offered an opportunity to correlate the absorption with the intermediary metabolism of cholesterol. A few experiments on the effect of fat on cholesterol metabolism in the liver are reported.

Materials and Methods.

White and piebald rats of an inbred laboratory strain were used. In most experiments the rats had initial body weights of 50—60 g. Each group consisted of 4 or 5 animals. The test animals and the corresponding control animals were drawn from the same litters. Male and female rats were present in the same proportion in the groups to be compared.

The rats were raised on a laboratory stock diet. Three to four days before the test period they were placed in metabolism cages and given

the experimental diets. These had a basic composition as follows: Casein 20 per cent, non-nutritive material (cellulose powder) 10 per cent, salt mixture 3 per cent, dry yeast 3 per cent, cane sugar 64 per cent. Into this basal diet cholesterol, fat and glycerol were incorporated, replacing equal amounts of sugar. The cholesterol was dissolved in ether and the solution poured over the ration under rapid mixing while the ether evaporated. Arachis oil was used as the fat source. A few groups of animals were given the cholesterol in the stock diet which consisted of: Dry skimmed milk 24 per cent, whole wheat 15 per cent, corn meal 20 per cent, whole rye 15 per cent, oatmeal 10 per cent, wheat germs 5 per cent, arachis oil 5 per cent, dry yeast 5 per cent, sodium chloride 0.5 per cent and calcium carbonate 0.5 per cent.

All animals were given 2 drops of cod liver oil per week. The rats on the fat free diets were also given linoleic acid (30 mg every other day) to meet the requirement for essential fatty acids. The animals were given free access to food and water at all times except in one experiment where pair-feeding was carried out.

The test periods lasted 7—17 days. During this period the food consumption was measured and the feces collected, carmine being used as a marker. Appropriate control groups received a cholesterol-free diet which was otherwise identical with that of the experimental groups. The "excess excretion" was obtained by subtracting the amount of cholesterol excreted by the control rats from the amount excreted by the rats fed cholesterol. The procedure involves the assumption that the same amount of endogenous cholesterol is excreted also when cholesterol is fed. The "apparent absorption" (cholesterol intake less "excess excretion") has been expressed in mg cholesterol per rat per day as well as in per cent of the cholesterol intake.

In a number of experiments the absorption was measured on individual rats. Since this is a time-consuming procedure only the mean absorption was determined in most experiments. The total food intake of each group of animals was measured and cholesterol analyses were carried out on samples of the pooled feces.

In some experiments the fecal sterol excretion was determined gravimetrically. The feces were dried at 100° C overnight and weighed, dispersed to a fine powder in a Waring blendor and extracted with ether for 24 hours in a Soxhlet apparatus. The ether soluble lipids were saponified for 1—2 hours with 10 per cent KOH in 95 per cent ethanol, the unsaponifiable fraction extracted with ether and the total sterols precipitated with digitonin as described by WINDAUS (1910). This method determines the coprosterol and dihydrocholesterol in addition to the cholesterol. However, in most of the experiments the colorimetric Schoenheimer-Sperry procedure was used for the determination of the fecal sterols. The ether soluble lipids were then treated with hot ethanol-acetone 1 : 1 and the total cholesterol content determined as described by SPERRY and WEBB (1950). In check runs known amounts of cholesterol added at various stages of the procedure were quantitatively recovered. When 8 fecal samples from rats fed cholesterol were analyzed with both methods, close agreement was found between the results,

the colorimetric procedure giving on the average 4.2 per cent lower values than the gravimetric method. This indicates that the coprosterol formation under the present dietary conditions was slight and that the Schoenheimer-Sperry procedure was adequate for the present purpose. Under similar dietary conditions KIM and IVY (1952) found excellent agreement between the gravimetric digitonin method and a colorimetric procedure based on the Liebermann-Burchard reaction.

In the experiments where the deposition of cholesterol in the liver was measured the animals were sacrificed by bleeding in ether anaesthesia and the livers were rapidly removed and weighed. The lipids were extracted and determined as described by BEST, LUCAS, PATTERSON and RIDOUT (1946) except that the Schoenheimer-Sperry procedure was used for the cholesterol determinations and the Fiske and SubbaRow method for determination of lipid phosphorus (HAWK, OSER and SUMMERSON 1947).

Results.

Variability. In the present study fairly uniform results were obtained in the experiments where the absorption was measured on individual rats. However, appreciable variations were found in the mean absorption of identically treated groups. The fact that greater variations were sometimes found between groups than within groups cautions against conclusions from anything but considerable and striking differences.

The absorption of cholesterol in the absence of fat. The absorption was measured in a number of groups given fat-free diet containing different levels of cholesterol. The results are compiled in table 1.

It is evident that appreciable fractions of the cholesterol consumed were not recovered in the feces. The data thus confirm the findings of BOLLMAN and FLOCK (1951) and of KIM and IVY (1952). However, the values here presented are higher than those reported by the latter authors. In contrast to the findings of KIM and IVY, the present data indicate that the percentage absorption of cholesterol declines with increasing concentrations of cholesterol in the food. However, they confirm the finding (KIM and IVY (1952)) that glycerol is without effect on cholesterol absorption.

The present results are at variance with the results of COOK (1936) which were obtained in similar balance experiments. Therefore, in an attempt to resolve this discrepancy, the cholesterol was mixed mechanically with the food in one case (group

Table 1. *The absorption of cholesterol on fat-free diets.*

Table 1.

The absorption of cholesterol on fat-free diets.

Group no.	No. of rats	Average body wt. g	Duration of period Days	Regimen: Diet	Regimen: Cholesterol %	Regimen: Glycerol %	Regimen: Linoleic acid	Cholesterol intake per day mg	Excretion per day mg	Excess excretion per day mg	Apparent absorption: mg	Apparent absorption: %
35	5	69	11	Basal diet	0.25	0	+	24.3	15.3	12.7	11.6	47.8
36	5	63	11		0	0	+		2.6			
21	5	73	14	» »	0.25	0	+	24.4	9.1	6.8	[1]17.6 ± 1.0	72.2 ± 1.7
22	5	82	14		0				2.4			
41	5	75	17	» »	0.25	0	+	21.9	9.9	7.5	14.4	65.8
22	5	82	14		0	0	+		2.4			
4a	5	73	14	» »	0.25	4	+	23.5	15.6	12.2	11.1 ± 1.3	47.7 ± 6.7
b	5	86	14		0	4	+		3.4			
5a	5	70	14	» »	0.25	4	—	23.1	15.8	12.0	11.2 ± 1.0	48.2 ± 2.0
b	5	71	14		0	4	—		3.8			
37	5	64	11	» »	0.25	4	+	21.4	12.7	9.6	11.8	55.1
38	5	67	11		0	4	+		3.1			
34	5	69	11	Chol. mixed mechanically	0.25	0	+	27.5	15.7	13.1	14.4	52.4
36	5	63	11		0	0	+		2.6			
83b	4	93	7	Basal diet	0.5	0	—	45.2	22.5	[3]10.3	25.9	57.3
3b	5	79	14	» »	1.0	0	—	94.3	[3]56.7	54.1	40.2 ± 1.5	43.3 ± 3.7
c	5	66	14		0	0	—		[3]2.6			
80a	4	60	7	» »	1.0	0	—	69.3	51.4	[3]48.2	21.1	30.5
27	5	74	14	» »	1.0	4	+	112.6	83.6	80.4	32.2 ± 4.2	28.8 ± 4.2
28	5	77	14		0	4	+		3.2			
53	4	62	8	»	1.0	4	—	66.3	40.7	38.3	28.0	42.2
22	5	82	14		0	0			2.4			
17	5	252	13	» »	1.0	4	+	140.0	91.2	87.4	52.6 ± 5.5	37.3 ± 2.2
18	5	251	13		0	4	+		3.8			
86a	4	67	7	Agar-agar used as roughage	1.20	0	—	96.5	56.5	54.0	42.5	43.0
84b	4	92	7		0	0			2.5			
85b	4	85	7	Basal diet	2.0	0	—	180.7	95.8	[2]92.6	88.1	48.8

[1] Standard error of the mean.
[2] 3.2 mg cholesterol used as "endogenous excretion".
[3] Sterol excretion determined gravimetrically.

Table 2.

The absorption of cholesterol in the presence of dietary fat.

Group no.	No. of rats	Average body wt. g	Duration of period Days	Regimen			Cholesterol intake per day mg	Excretion per day mg	Excess excretion per day mg	Apparent absorption	
				Diet	Cholesterol %	Fat %				mg	%
89	4	133	7	Basal diet	0.15	10	17.0	6.2	1.7	15.3	90.0
88	4	84	7	+ fat	0	10		4.5			
23	5	84	14	» »	0.25	1	27.8	10.3	7.9	19.8 ± 0.7	71.4 ± 1.5
24	5	74	14		0	1		2.4			
38b	5	99	14	» »	0.25	5	26.5	5.6	3.0	23.5	88.7
74	4	56	11		0	5		2.6			
50	4	68	11	» »	0.25	5	14.1	3.7	1.1	13.0	92.2
51	4	66	11		0	5		2.6			
33	5	74	14	Stock diet	0.25	5	22.7	9.3	2.0	20.7	91.2
32	5	77	14		0	5		7.3			
10	5	93	14	Basal diet + fat	0.25	10	27.1	8.6	4.1	22.9 ± 1.3	84.6 ± 3.0
9	5	84	14	(chol. mixed mech.)	0	10		4.5			
11	5	83	14	Basal diet + fat	0.25	10	24.7	6.2	1.7	23.0 ± 1.3	92.7 ± 1.3
9	5	84	14		0	10		4.5			
90	6	140	7	» » »	0.25	10	29.3	11.7	7.2	22.1	75.5
88	4	84	7		0	10		4.5			
91	2	140	7	» » »	0.5	10	62.0	12.6	8.1	53.9	86.8
88	4	84	7		0	10		4.5			
25	5	78	14	» » »	0.5	15	46.1	16.4	11.2	34.9 ± 3.0	75.2 ± 4.6
26	5	74	14		0	15		5.2			
42	5	76	17	» » »	0.5	15	36.6	10.5	5.3	31.3	85.5
26	5	74	14		0	15		5.2			
52	4	64	11	» » »	1.0	5	55.7	28.7	26.1	29.6	53.7
74	4	56	11		0	5		2.6			
75	4	57	11	» » »	1.0	5	60.7	38.6	36.0	24.7	40.7
74	4	56	11		0	5		2.6			
83a	4	63	7	» » »	1.0	10	71.8	44.7	40.2	31.8	44.3
88	4	84	7		0	10		4.5			
1	5	351	14	» » »	1.0	15	146.3	70.1	62.3	83.9 ± 5.9	57.5 ± 4.4
2	5	341	14		0	15		7.8			

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Table 3.

The effect of dietary fat on cholesterol absorption.

Per cent cholesterol in diet	Cholesterol absorption in per cent			
	Per cent arachis oil in diet			
	0	5	10	15
0.25	[1]55.6 (7) 47.7—72.2	90.7 (3) 88.7—92.2	84.3 (3) 75.5—92.7	—
0.50	57.3 (1)		86.8 (1)	80.4 (2) 75.2—85.5
1.00	36.4 (5) 28.8—43.3	47.2 (2) 40.7—53.7	44.3 (1)	57.5 (1)

[1] The average value for the groups studied. The figure in parenthesis gives the number of groups, while the figures below the average value give the range.

34) and in another (group 86 a) the cellulose powder was replaced by agar-agar, to approach more closely the experimental conditions used by COOK. However, none of these modifications appeared to alter our results.

In order to rule out the possibility that the added linoleic acid might have stimulated cholesterol absorption, linoleic acid was omitted from the diet in a few groups. From a comparison between groups 4 and 5 (table 1) it is clear that the small amount of linoleic acid usually given was without measurable effect on the cholesterol absorption.

It will be noted that in group 3 b where the sterol excretion was measured gravimetrically, the cholesterol absorption was found to be at least as great as in the groups where the Schoenheimer-Sperry method was used. Thus there can be no doubt that in rats cholesterol is readily absorbed in the absence of dietary fat.

The effect of fat on cholesterol absorption. The data on the absorption of cholesterol in animals given fat in the diet are detailed in table 2. The effect of the added fat becomes clear when table 2 is compared with table 1. Some of the relevant data have been assembled in a condensed table to facilitate the comparisons.

A given concentration of fat increased the absorption of cholesterol in some cases, but not in others (table 3). Thus with 0.25 per cent cholesterol in the diet, the addition of both 5 and 10

Table

The effect of dietary fat on the de-

The rats were fed cholesterol, in the presence

Group no.	No. of rats	Average body wt. g	Additions to basal diet		Cholesterol absorption per day[1] mg	Liver weight	
			Cholesterol %	Fat %		Wet wt. mg	Fat-free dry residue mg
42	5	114	0.5	15	31.3	5,040	907
26	5	142	0	15		6,120	1,480
27	5	132	1.0	0	32.2 ± 4.2	6,040	1,320
28	5	135	0	0		5,000	1,172

[1] The absorption was measured in periods of 17 and 14 days for the animals
[2] Lipid P × 25.2.
[3] Total lipids — (phospholipids + free cholesterol + cholesterol esters).
[4] (Total cholesterol — free cholesterol) × 1.68.

per cent arachis oil increased the absorption of cholesterol. However, these same levels of fat did not convincingly increase the absorption when 1 per cent cholesterol was given. When, on the other hand, the level of fat was raised to 15 per cent, the absorption appeared to be definitely increased also with 1 per cent cholesterol in the diet. The present data thus support the conclusion reached by KIM and IVY (1952) that dietary fat does only increase cholesterol absorption when it is present in relatively large amounts compared to cholesterol. In the experiments here reported the lowest ratio of fat to cholesterol giving a demonstrable effect was 15 : 1, whereas KIM and IVY found a stimulating effect of fat when the ratio was 24 : 1, but no effect when it was only 8 : 1.

The effect of fat on cholesterol metabolism in the liver. Evidence is accumulating that the intermediary metabolism of cholesterol is influenced by the level of fat in the diet (ECKSTEIN 1938, KEYS, MICKELSEN, MILLER and CHAPMAN 1950, HILDRETH, MELLINKOFF, BLAIR and HILDRETH 1951, ALFIN-SLATER, SCHOTZ, SHIMODA and DEUEL 1952, ANDERSON and KEYS 1953). In the present investigation the effect of fat a) on the deposition in the liver of absorbed cholesterol and b) on the removal of stored cholesterol, was studied.

In the first experiment the deposition of cholesterol in the liver was measured in rats given 1 per cent cholesterol without fat,

4.

position of cholesterol in the liver.
and absence of dietary fat, for 7 weeks.

Liver lipids						Serum cholesterol mg per 100 ml.
Total lipids mg	Phospholipids[2] mg	Neutral fat[3] mg	Cholesterol			
			Free mg	Total mg	Chol. esters[4] mg	
872	126	212	22	326	511	111 ± 37
383	175	184	12	19	12	
705	154	293	17	160	240	83 ± 13
332	169	144	10	15	8	

given the fat-containing and the fat-free diet respectively.

and 0.5 per cent cholesterol with 15 per cent fat in the diet respectively. These levels of cholesterol were chosen since it was reason to expect from previous absorption measurements that approximately equal amounts of cholesterol would be absorbed under these conditions. The absorption of cholesterol was measured. After 7 weeks of feeding the animals were sacrificed and lipid analyses of the livers carried out (table 4).

Typical fatty livers with the accumulation of substantial amounts of cholesterol esters were found in both cholesterol-fed groups. Roughly twice as much cholesterol was deposited in the livers of the rats given the fat-containing diet as in the rats on the fat-free diet, although the absorption measurements indicate that nearly identical amounts were absorbed in the two groups. These results indicate that the effect of dietary fat on the deposition of cholesterol in the liver cannot be explained entirely on the basis of its effect on the absorption. Additional data supporting this conclusion were obtained in experiments where the effect of bile acids on cholesterol absorption and deposition was studied in the absence and presence of fat (PIHL 1955).

Since the present data on the effect of fat on the deposition of cholesterol are of limited extent, it is of interest to recall the observations by LOIZIDES (1937). In his experiments a progressive increase in cholesterol deposition occurred in the livers of rats fed cholesterol with increasing levels of dietary fat. However, as he

Table

The effect of dietary fat on choles-

The incorporation of 1-C^{14} acetate into cholesterol, fatty acids and respiratory and were subsequently given, for the last 8 days prior to the

Rat No.	Final body wt.	Food intake[1] g	Diet[2]
376	135	87	Basal diet + 2 per cent arachis oil
377	141	84	» » » » » » » »
375	120	74	» » » » » » » »
379	128	67	Basal diet + 30 per cent arachis oil
378	154	61	» » » » » » » »
382	122	56	» » » » » » » »

[1] Pair-feeding carried out in such a way as to insure the same calorie intake
[2] Diet during the last 8 days.
[3] Pooled sample.

found as much as a fourfold increase in the deposition when the ratio of fat to cholesterol was raised from 5.5 : 1 to 20.5 : 1, it appears likely that a substantial part of the observed effect in his experiments was due to a metabolic action of the fat.

While the preceding experiment indicated that dietary fat promotes the deposition of absorbed cholesterol, ALFIN-SLATER, SCHOTZ, SHIMODA, and DEUEL (1952) have obtained evidence that dietary fat promotes the disappearance of stored cholesterol. When rats with "cholesterol fatty liver" were placed on cholesterol-free diets, low and high in fat, the decrease in the stored cholesterol was more marked in the animals on the high-fat diet. Since these results were difficult to reconcile with the results here reported, the following experiment was carried out (table 5). Deposition of cholesterol in the liver was produced by feeding 1 per cent cholesterol in stock diet for 34 days. The rats were then separated into two groups which were given cholesterol-free basal diet containing 2 per cent and 30 per cent arachis oil respectively. In order to insure the same calorie intake in the two groups the animals in this experiment were "pair-fed" in such a way that the rats given the high-fat diet consumed about 70 per cent of the amount of those on the low-fat diet. After 8 days' feeding the animals were sacrificed, and pooled samples of the livers were analyzed for the total cholesterol content. Slices from

5.

terol metabolism in the liver.

CO_2 by surviving liver slices. The rats had been fed 1 % cholesterol for 34 days experiment, cholesterol-free diets, high and low in fat respectively.

Liver			Per cent of added C^{14} recovered in		
Weight g	Per cent of body wt.	Chol cont. %	Chol.	Fatty acids	CO_2
6.41	4.75		0.04	0.32	48.9
6.24	4.42	1.49[3]	0.18	1.07	52.6
6.20	5.17		0.09	2.74	52.3
5.25	4.10		0.63	0.47	50.6
7.33	4.76	1.08[3]	0.04	0.37	49.5
5.87	4.81		1.86	0.72	43.7

in the two groups.

the individual livers were incubated with 1-C^{14} acetate and the incorporation of C^{14} into cholesterol, fatty acids and respiratory CO_2 was measured according to procedures described elsewhere (PIHL 1955).

As seen from table 5 the cholesterol content of the livers was the lower in the animal fed the high-fat diet, in agreement with the findings of ALFIN-SLATER et al. (1952). Furthermore, the incorporation of acetate into cholesterol was distinctly greater in these livers. This result is likewise consistent with data obtained by ALFIN-SLATER et al. In their experiments also the cholesterol synthesis, as measured *in vivo* with the aid of heavy water, was greater in the animals given the high fat diet. The results of the two studies demonstrate an effect of dietary fat on the disappearance of stored cholesterol which can not be explained by an effect of the fat on the hepatic cholesterol synthesis.

Discussion.

The methods used in the study of cholesterol absorption all suffer from inherent shortcomings. The use of the balance method is complicated by the possibility that part of the cholesterol not recovered in the feces may have been altered or destroyed by bacterial activity in the intestinal tract. The bacterial conversion

of cholesterol to coprosterol, which is known to vary with the diet (MÜLLER 1900, DAM 1934, ROSENHEIM and WEBSTER 1941) was found to be of no consequence in this study, as was the case in the study by KIM and IVY (1952). It has recently been found (WAINFAN, HENKIN, RITTENBERG, and MARX 1954) that appreciable destruction of cholesterol may take place when it is incubated with rat feces *in vitro*, while the significance of intestinal cholesterol destruction in rats *in vivo* is not known. However, where the effect of various factors on cholesterol absorption is tested in balance experiments, the results will be valid even if some intestinal destruction of cholesterol does take place, as the destruction will operate also in the control groups.

The fact that cholesterol can be absorbed in the absence of dietary fat is of considerable interest with respect to its mechanism of absorption. Even on a fat-free diet, however, the intestinal tract is not entirely devoid of fat, since fat of endogenous origin will always be present. Whether this endogenous fat is a decisive factor for the cholesterol absorption observed in animals on fat-free diets can not be stated with certainty. However, the finding that relatively large amounts of cholesterol were absorbed under these conditions, and the further fact that the absorption increased with increasing levels in the diet, indicate that the endogenous fat does not account for the cholesterol absorption observed on fat-free diets.

The data presented in this paper support the theses (KIM and IVY 1952) that dietary fat stimulates cholesterol absorption only when it is present in large amounts relative to cholesterol. Since they found that free fatty acids stimulated cholesterol absorption to a greater extent than an equivalent amount of fat, while glycerol was without effect, the effect of fat was attributed entirely to liberated free fatty acids and the quantitative relationships were thought to reflect incomplete hydrolysis of the fat in the intestinal tract. It appears, however, that if the free fatty acids as such must be present in certain minimum amounts relative to cholesterol in order to be effective, the fact that fat is needed in high proportions might be explained irrespective of whether fat is completely or only partially hydrolyzed in the intestine.

The data here reported on the metabolism of cholesterol in the liver gave results which appear conflicting. On the one hand did dietary fat appear to increase the deposition of absorbed

cholesterol, while in the other it seemed to promote the removal of stored cholesterol from the liver of animals on a cholesterol-free diet. These contrasting effects of dietary fat under different experimental conditions indicate that dietary fat influences intermediary cholesterol metabolism by more than one mechanism.

Summary and Conclusions.

The absorption of cholesterol has been studied in balance experiments on intact rats fed cholesterol together with different levels of arachis oil.

Cholesterol was found to be readily absorbed on a diet devoid of fat. Extensive deposition of cholesterol esters was found in the livers of rats given cholesterol in a fat-free diet for 7 weeks.

Dietary fat was found to increase cholesterol absorption when the fat was present in large amounts as compared to cholesterol, confirming qualitatively the observations of previous authors. The smallest ratio of fat to cholesterol where a facilitating effect of fat was observed, was 15 : 1.

The effect of dietary fat on the deposition of absorbed cholesterol was studied in experiments so designed that equal amounts of cholesterol were absorbed in groups fed diets with 15 per cent fat and no fat respectively. Roughly twice as much cholesterol was deposited in the livers of the rats given the fat-containing diet. On the other hand, in rats with cholesterol deposited in the liver dietary fat appeared to promote the removal of cholesterol from the liver, when the rats were given cholesterol-free diets.

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The Nutrition Research Institute, University of Oslo, Norway.

On the Mode of Action of Fatty Acids in Cholesterol Absorption.[1]

Cholesterol Studies IV.

By

ALEXANDER PIHL.[2]

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Recent work has demonstrated that although dietary fat is not obligatory in the absorption of cholesterol in rats, it definitely stimulates the absorption, provided it is present in the diet in large amounts compared to cholesterol (KIM and IVY 1952, PIHL 1955 a). Since the free fatty acids have been found to stimulate cholesterol absorption to a greater extent than an equivalent amount of fat, while glycerol is without effect, the facilitating effect of fat has been attributed entirely to the free fatty acids (KIM and IVY 1952).

The explanation most commonly offered for the stimulating effect of fatty acids on cholesterol absorption is that they increase the absorption by esterifying the cholesterol. This view is predominantly based on the fact that cholesterol is in part esterified during its passage from the intestinal lumen to the lymph and blood (FRASER and GARDNER 1910, MUELLER 1915, SANO 1924, FRÖLICHER and SÜLLMAN 1934, BOLLMAN and FLOCK 1951, CHAIKOFF, BLOOM, SIPERSTEIN, KIYASU, REINHARDT, DAUBEN and EASTHAM 1952). The demonstration by numerous authors

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[2] Fellow of the Norwegian Cancer Society.

Present address: Norsk Hydro's Institute for Cancer Research, The Norwegian Radium Hospital.

that pancreas and intestinal mucosa contain enzymes catalyzing the esterification of free cholesterol and the splitting of cholesterol esters, and the further fact that various cholesterol esterase preparations are activated by bile salts have been taken as additional evidence for the esterification hypothesis (MUELLER 1916, NEDSWEDSKI 1935, SCHRAMM and WOLFF 1940, LEBRETON and PANTALEON 1947, NIEFT and DEUEL 1949, SWELL, BYRON and TREADWELL 1950, SWELL, FIELD and TREADWELL 1953).

However, observations are available which are not easily brought in harmony with the esterification hypothesis. Thus BOLLMAN and FLOCK (1951) found that also in rats absorbing cholesterol in the absence of dietary fat did the major increase in the intestinal lymph cholesterol occur in the ester fraction. Even more important, FAVARGER and METZGER (1952) were unable to demonstrate esterification of cholesterol in the intestinal lumen or the intestinal wall of rats in experiments where labelled cholesterol was fed. On the contrary, evidence was obtained that cholesterol esters are split in the intestine and that this process continues in the intestinal wall.

In the present paper experiments on the rôle of fatty acids in cholesterol absorption are reported. The results obtained seem to indicate that the stimulating effect of fatty acids on cholesterol absorption is unrelated to the esterification of cholesterol known to occur during its transport from the intestinal lumen to the lymph.

Experimental.

The absorption of cholesterol was measured in balance experiments on young rats given cholesterol in free form together with free fatty acids, and the cholesterol esters of the same fatty acids respectively. The experimental procedure as well as the basal diet and the analytical methods have previously been described (PIHL 1955 a).

The fatty acids used were oleic and stearic acid. They were given in a concentration of 9 per cent in the diet together with 1 per cent cholesterol. The cholesterol esters were given in amounts corresponding to 1 per cent cholesterol. Since the animals given free fatty acids consumed less food than those given the cholesterol esters, the groups to be compared were pair-fed in two instances. In one experiment the absorption of cholesterol acetate was studied. The animals were given 0.25 per cent, calculated as free cholesterol, in the diet.

Total and free cholesterol, and in some cases lipid phosphorus, were determined in feces. The ether soluble lipids were redissolved in benzene since considerable amounts of phospholipids were excreted in the animals

fed free fatty acids. The total cholesterol content was determined after evaporation of the benzene. As the presence of relatively large amounts of phospholipids or cholesterol esters tended to interfere with the determination of free cholesterol, aliquots of the benzene solutions were evaporated, the residues were extracted with hot acetone-ethanol 1 : 1, and the determinations were carried out on the cooled filtered extracts.

Results.

Absorption. The effect of 9 per cent oleic or stearic acid on the absorption of cholesterol can be seen from table 1. It is evident that whereas oleic acid clearly stimulated the absorption, no effect was observed with stearic acid under the present conditions. This different effect of the unsaturated and the saturated fatty acid is in principal agreement with the finding of KIM and IVY (1952) that oleic acid stimulated cholesterol absorption more strongly than palmitic acid. Similar results have been obtained in a recent study where the blood cholesterol level was used as indicator for the cholesterol absorption (SWELL and FLICK 1953). It will be noted[1] that when 0.5 per cent cholic or desoxycholic acid was added to the diet containing 9 per cent oleic acid, the absorption of cholesterol was not greater than in group 94 where cholesterol and oleic acid only was fed.

If oleic acid stimulates the absorption of cholesterol by esterifying the cholesterol in the intestinal lumen, this would imply that under comparable conditions cholesterol is more efficiently absorbed in the form of cholesterol oleate than as free cholesterol. However, this was not found to be the case. In the groups where cholesterol oleate was given in the absence of free fatty acids (groups 80 b and 95) the absorption of cholesterol was 49.0 and 30.9 per cent respectively. Under similar conditions the absorption of free cholesterol was found to be 29—43 per cent (PIHL 1955 a). The data therefore strongly indicate that cholesterol oleate is not absorbed more readily than free cholesterol. Appreciably greater absorption was found in the groups fed cholesterol with 9 per cent oleic acid than in the groups fed cholesterol oleate only. The difference is particularly evident when the pair-fed groups are compared. This result demonstrates clearly that cholesterol oleate formation prior to absorption can not explain the stimulating effect of the oleic acid. However, in the groups fed free oleic acid, excess free acid would be present even if complete

[1] See PIHL 1955 b.

Table

The absorption of cholesterol when given with free fatty

Group no.	No. of animals	Additions to basal diet			Duration of period. Days
			Chol. %	Free fatty acids %	
80 a	4	Cholesterol	1	—	7
81 a	4	Chol. + oleic acid..	1	9	7
84 a	4	Chol. + oleic acid + cholic acid....	1	9	7
85 a	4	Chol. + oleic acid + desoxycholic acid	1	9	7
80 b	4	Cholesterol oleate ..	[1]1	—	7
82 a	4	Cholesterol oleate + oleic acid.....	[1]1	[4]9	7
94[3]	4	Chol. + oleic acid..	1	9	4
95	4	Cholesterol oleate ..	[1]1	—	4
81 b	4	Chol. + stearic ac..	[1]1	9	7
82 b	4	Chol. stearate	1	—	7
92[3]	4	Chol. + stearic acid	1	9	4
93	4	Chol. stearate	[1]1	—	4
29	5	Chol. acetate	[2]0.25	—	14

[1] Calculated as free cholesterol.
[2] 3.2 mg cholesterol per day (group 28) has been used as "endogenous excretion"
[3] "Pair-fed".
[4] Total amount of oleic acid (free and bound).

esterification of cholesterol occurred. Therefore, in order to measure the absorption of free and esterified cholesterol under more comparable conditions, cholesterol oleate in the presence of free oleic acid was given (group 82 a). It is evident that also under these conditions did the absorption of cholesterol oleate not exceed the absorption of free cholesterol. Despite the considerable variability in the present experiments, the data demonstrate clearly that cholesterol oleate is not preferentially absorbed.

It further appears from table 1 that when cholesterol was given as the stearate, it was absorbed to the same or to a lesser extent than when given as free cholesterol. This is not surprising since free stearic acid did not stimulate the absorption. It will be noted that cholesterol was readily absorbed also when fed in the form of acetate.

Esterification and hydrolysis. In order to obtain further information on the significance of esterification of cholesterol in the intestine, the ratio of free to total cholesterol was determined in the feces. The results are presented in table 2.

1.

acids and when given as the corresponding esters.

Average body wt. g	Chol. intake per day mg	Excretion per day[1] mg	Excess excretion per day mg	Apparent absorption	
				mg	%
60	69.3	51.4	48.2	21.1	30.5
61	62.1	31.7	28.5	33.6	54.1
60	60.7	16.8	13.6	47.1	77.6
57	59.3	22.8	19.6	39.7	66.9
91	88.2	48.2	45.0	43.2	49.0
66	70.0	33.9	30.7	39.3	56.1
98	98.8	22.6	19.4	79.4	80.3
97	96.3	69.7	66.5	29.8	30.9
85	78.9	52.6	49.4	29.5	37.7
96	91.4	63.0	59.8	31.6	34.5
90	71.9	48.1	44.9	27.0	37.6
90	81.3	64.6	61.4	19.9	24.5
73	25.1	15.4	12.2	12.9	51.4

for all groups in this table.

In the rats on cholesterol- and fat-free diet the endogenous cholesterol in feces was present predominantly in free form. When cholesterol was added to the fat-free diet, the fecal cholesterol consisted of free cholesterol exclusively, as might be expected. The further addition of 10 per cent arachis oil or 9 per cent oleic acid did not result in the excretion of measurable amounts of cholesterol esters. Since cholesterol esters are not preferentially absorbed, the results indicate that the formation of cholesterol oleate must have been negligible under these conditions. The further addition of cholic or desoxycholic acid to the rats given cholesterol and oleic acid also did not result in the excretion of cholesterol esters. Thus the view that dietary bile acids increase cholesterol absorption by stimulating esterification of cholesterol (SCHRAMM and WOLFF 1940, SWELL, FLICK, FIELD and TREADWELL 1953) finds no support in the present data. In the rats given stearic acid, 20—30 per cent of the cholesterol was found to be excreted in the esterified form.

In the animals fed cholesterol esters substantial fractions of the cholesterol were excreted in free form. This was the case also

Table 2.

Free and esterified cholesterol in feces.

Group no.	Additions to basal diet	Cholesterol %	Fat %	Free fatty acids %	Free cholesterol in per cent of total cholesterol
28	None	0	0	0	80.6
36	None	0	0	0	85.7
83 b	Cholesterol	0.5	0	0	100.0
80 a	»	1.0	0	0	99.8
85 b	»	2.0	0	0	100.0
83 a	Chol. + arachis oil.	1	10	0	99.3
81 a	Chol. + oleic acid..	1	0	9	100.0
94	» ..	1	0	9	97.0
84 a	Chol. + oleic acid + cholic acid[1].....	1	0	9	99.7
85 a	Chol. + oleic acid + desoxycholic acid[1]	1	0	9	98.4
81 b	Chol. + stearic acid	1	0	9	67.8
92	»	1	0	9	80.4
80 b	Cholesterol oleate ..	[2]1	0	0	44.0
95	» ..	[2]1	0	0	43.9
82 a	Cholesterol oleate + oleic acid	[2]1	0	[3]9	89.4
82 b	Cholesterol stearate	[2]1	0	0	33.7
93	»	[2]1	0	0	53.7
29	Cholesterol acetate.	[2]0.25	0	0	77.0

[1] 0.5 per cent in the diet.
[2] Calculated as free cholesterol.
[3] Total amount of oleic acid (free and bound).

when cholesterol stearate or cholesterol acetate was given. The results demonstrate that extensive splitting of cholesterol esters may occur in the intestine, in agreement with previous findings (FAVARGER and METZGER 1952). Since it is well established that pancreatic juice of rats contains cholesterol esterases, the splitting and formation of cholesterol esters observed in the present experiments most likely were effected by these enzymes. Whether bacterial enzymes were active in this respect can not be said.

Altogether the present data indicate that the conditions in the intestine of the rat favour hydrolysis of cholesterol esters.

Phospholipid excretion. In the animals fed free fatty acids the ether-soluble lipids were not completely soluble in acetone-ethanol. When 4 volumes of acetone were added to an ether solution of the lipids, a white precipitate which rapidly turned brown was

Table 3.

Lipid phosphorus excretion in feces.

Group no.	Average body wt. g	Additions to basal diet	Cholesterol %	Fatty acids %	Lipid P mg per day
84 b	92	None	0	0	0.02
85 b	85	Cholesterol	2.0	0	0.06
80 b	91	Cholesterol oleate	[1]1.0	0	0.05
95	97	» »	[1]1.0	0	0·04
93	90	Cholesterol stearate ...	[1]1.0	0	0.04
81 b	85	Cholesterol + stearic acid	1.0	9	0.50
92	90	Cholesterol + stearic acid	1.0	9	0.24
94	98	Cholesterol + oleic acid	1.0	9	3.42

[1] Calculated as cholesterol.

formed. The precipitate could easily be redissolved in ether. The solubility characteristics indicated the presence of increased amounts of phospholipids in the feces of these rats. The lipid phosphorous excretion was therefore measured in some of the groups. The results given in table 3 demonstrate that the administration of free fatty acids strongly increased the lipid phosphorous excretion and that oleic acid was more active than stearic acid in this respect. The results are qualitatively in agreement with observations by KIM and IVY (1952).

Discussion.

The present absorption measurements demonstrate that the stimulating effect of free fatty acids on cholesterol absorption can not be explained by the formation of cholesterol esters prior to absorption, as assumed by several investigators (MUELLER 1916, SCHRAMM and WOLFF 1940, LEBRETON and PANTALEON 1947, SWELL, FLICK, FIELD and TREADWELL 1953). In fact, evidence that esterification of cholesterol occurs in the intestinal tract was only obtained with stearic acid which did not stimulate the absorption. This lack of correlation between the ability of free fatty acids to esterify cholesterol and their ability to facilitate cholesterol absorption, suggests that esterification in the intestinal lumen by extracellular cholesterol esterases is not in-

volved in the absorption of cholesterol. On the contrary, the extensive hydrolysis of the administered cholesterol esters observed in the present experiments suggests that the main function of the pancreatic cholesterol esterase may be to split dietary cholesterol esters. The splitting activity appears to be rather unspecific since cholesterol acetate, an ester which has not been found in nature, was extensively hydrolyzed. The lack of specificity may explain the observation by SCHÖNHEIMER and HUMMEL (1930) that cholesterol was absorbed in mice also when given as the oxalate ester.

Since the free fatty acids do not exert their effect on cholesterol absorption by esterifying cholesterol in the intestinal tract, their rôle in the solubilization or dispersion of cholesterol should be considered. The ability of free fatty acids to increase the excretion of phospholipids may be of significance in this respect. The origin of these phospholipids is conceivably the bile since — at least in man — bile contains high concentrations of lecithin (ISAKSSON 1951, POLONOVSKI and BOURRILLON 1952). The presence of the lecithin seems to be an important factor in the solubilization of cholesterol in bile. In fact, evidence has been presented to show that cholesterol is kept in solution in bladder bile in the form of a complex where lecithin, bile salts and cholesterol are present in definite proportions (ISAKSSON 1951, 1953—1954). In view of this the possibility should be considered that an increased concentration of phospholipids in the intestinal tract is part of the mechanism whereby free fatty acids stimulate cholesterol absorption.

Summary and Conclusions.

In balance experiments on rats oleic acid (9 per cent in the diet) was found to increase the absorption of cholesterol (1 per cent in the diet) while stearic acid had no effect. Cholesterol oleate and cholesterol stearate were not absorbed to a greater extent than was free cholesterol under comparable conditions. Cholesterol was readily absorbed also when given as the acetate ester.

Cholesterol esters were detected in feces when free cholesterol with stearic acid was fed, but not when oleic acid was given. Addition of bile acids did not alter this result. Administered cholesterol esters, the acetate ester inclusive, were extensively

split in the intestine. The results demonstrate that the stimulating effect of free fatty acids on cholesterol absorption is not due to formation of cholesterol esters prior to absorption and that the conditions in the intestinal tract of the rat favour hydrolysis of cholesterol esters.

Feeding of free fatty acids stimulated strongly the excretion of phospholipids in feces. Oleic acid was more active than stearic acid in this respect.

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The Nutrition Research Institute, University of Oslo, Norway.

The Effect of Bile Acids on Cholesterol Absorption, Deposition and Synthesis in the Rat.[1]

Cholesterol Studies V.

By

ALEXANDER PIHL.[2]

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When bile acids are fed to animals given cholesterol in the diet, higher levels of cholesterol will be found in the blood and liver than when cholesterol is given without bile acids. This fact was first demonstrated by SCHÖNHEIMER and his pupils, and has later been confirmed by numerous authors in experiments on different animal species. SCHÖNHEIMER (1924) observed in short term experiments that when rabbits were given desoxycholic acid in addition to cholesterol and fat, the consequent rise in the blood cholesterol level was far greater than in the control animals not given bile acid. He therefore concluded that desoxycholic acid addition had stimulated the absorption of cholesterol. Subsequently LOEFFLER (1928) and HUMMEL (1929) studied, in feeding experiments on mice and rabbits, the effect of different bile acids on the deposition of cholesterol in the liver. Cholic acid was found to increase the deposition of dietary cholesterol more strongly than did desoxycholic acid. Glycocholic acid and apocholic acid likewise strongly enhanced cholesterol deposition. Dehydrocholic acid, however, had no definite effect. The results were attributed

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[2] Fellow of the Norwegian Cancer Society.

Present address: Norsk Hydro's Institute for Cancer Research, The Norwegian Radium Hospital.

to a stimulating effect of the bile acids on cholesterol absorption, an interpretation offered also by subsequent authors (MEMBER, BRUGER and OPPENHEIM 1944, KIM and IVY 1952, SWELL, FIELD, FLICK and TREADWELL 1953, ROSENMAN, BYERS and FRIEDMAN 1953).

Direct measurement of the effect of bile acids on cholesterol absorption were, however, not carried out until recently. In a balance experiment KIM and IVY (1952) found no stimulating effect of desoxycholate on cholesterol absorption in rats. On the other hand, in the experiments of FRIEDMAN, BYERS and SHIBATA (1953) cholic acid addition increased cholesterol absorption. However, simultaneous measurements of the effect of bile acids on the absorption and deposition of cholesterol, permitting correlations to be made, have previously not been carried out.

Cholesterol is known to be both synthesized and degraded in the liver. The possibility that bile acid ingestion might affect these processes did not seem inconceivable. Evidence that other aspects of cholesterol metabolism than absorption may be affected can, in fact, be found in the literature. Thus HUMMEL (1929) observed that feeding of different bile acids to normal mice led to a definite increase in the hepatic cholesterol content, even in the absence of dietary cholesterol. Furthermore, when mice with cholesterol deposited in the liver were fed certain bile acids on a cholesterol-free diet, the normal disappearance of cholesterol was prevented. In view of these facts a further investigation of the rôle of bile acids in cholesterol metabolism seemed warranted.

In the present experiments the effect of different bile acids on cholesterol absorption, deposition and synthesis has been studied. Evidence has been obtained that the increased cholesterol deposition in rats fed bile acids can not be adequately explained by a stimulating effect on cholesterol absorption as assumed by previous authors. The present data indicate that ingestion of bile acids influences the intermediary metabolism of cholesterol, probably by interfering with its catabolism in the liver.

Experimental.

Young rats, males and females in equal numbers, were used. The analytical methods and the general procedure for the measurement of the absorption and deposition of cholesterol have previously been described (PIHL 1955). The absorption periods lasted 7—17 days. The

bile acids (cholic, desoxycholic and dehydrocholic acid,[1] all at a level of 0.5 per cent) were given in three different diets, viz. the basal diet previously described (PIHL 1955) and the same diet with the addition of 5 per cent arachis oil or 9 per cent oleic acid respectively. The bile acids were given in the presence and absence of cholesterol, the latter groups being included to detect possible effects of the bile acids on the endogenous cholesterol metabolism. The control groups received identical diets except that the bile acids were omitted. The arrangements of the groups and the procedure for calculating the results are most readily seen from the tables.

The effect of cholic acid feeding on the hepatic cholesterol synthesis was studied by measuring the incorporation of 1-C^{14}-acetate into cholesterol in surviving liver slices. The incubation was carried out in wide-necked Erlenmeyer flasks. 700 mg of slices from individual livers were incubated in 7 ml Krebs-Ringer phosphate buffer, pH 7.4, containing 1.56 μM of 1-C^{14}-acetate. The atmosphere was oxygen and the temperature 38° C. The respiratory CO_2 was collected in a suspended chamber containing sodium hydroxide and a folded filter paper strip. After the incubation, which lasted for three hours, the flasks were rapidly cooled and 1 ml 1 N H_2SO_4 was injected to stop the reaction and to liberate bound carbon dioxide. The flasks were left overnight to insure complete absorption of the CO_2 which was subsequently precipitated as barium carbonate. To the flasks were added sufficient KOH and ethanol to give a concentration of 10 per cent KOH in 50 per cent ethanol, and the flasks were refluxed on a boiling water bath for three hours. The unsaponifiable fraction was extracted with ether, and the cholesterol precipitated as the digitonide (WINDAUS 1910). The washings from the ether extracts and the alkaline water phase were combined, and the free fatty acids liberated by adding sulfuric acid. The fatty acids were extracted with petrol ether, washed twice with 5 per cent acetic acid and twice with water and subsequently dried and weighed. The cholesterol digitonide and the fatty acids were oxidized to carbon dioxide and converted to $BaCO_3$ by conventional methods (CALVIN, HEIDELBERGER, REID, TOLBERT and YANKWICH 1949). Barium carbonate samples were prepared in standard cupped planchets and the radioactivity determined with a thin window G. M. counter. The data were corrected, when necessary, to give the activity of infinitely thick samples. The results have been expressed as the per cent of the added radioactivity recovered in the labelled constituent.

Results.

Absorption. The effect of the bile acid ingestion on the absorption of cholesterol can be seen in table 1.

In the group of rats fed cholic acid with 0.25 per cent cholesterol in fat-free diet the percentage absorption was greater than in the

[1] Nutritional Biochemicals Co.

The effect of bile acid ingestion on cholesterol absorption.

Group no.	No. of rats	Average body wt. g	Duration of period. Days	Additions to basal diet: Bile acid	Additions to basal diet: Free fatty acids[1] %	Additions to basal diet: Fat %	Additions to basal diet: Cholesterol %	Cholesterol intake per day mg	Excretion per day mg	Excess excretion per day mg	Apparent absorption: mg	Apparent absorption: %
41	5	75	17	None	0	0	0.25	21.8	9.9	6.7	15.1	69.2
28	5	77		»			0		3.2			
39	5	67		Cholic acid			0.25	17.8	4.8	2.6	15.2	85.4
40	5	62		» »			0		2.2			
53	4	62	8	None	0	0	1.0	66.3	40.7	37.5	28.8	43.4
28	5	77		»			0		3.2			
43	4	64		Cholic acid			1.0	69.0	46.8	44.6	24.4	35.4
40	5	62		» »			0		2.2			
45	4	61		Desoxycholic acid			1.0	59.3	39.9	37.4	21.9	36.9
54	4	57		» »			0		2.5			
47	4	66		Dehydrocholic acid			1.0	61.9	42.5	39.7	22.2	35.9
48	4	67					0		2.8			
52	4	65	11	None	0	5	1.0	55.7	28.7	26.1	29.6	53.1
51	4	66		»			0		2.6			
75	4	57		None			1.0	60.7	38.6	36.0	24.7	40.7
74	4	56		»			0		2.6			
44	4	62		Cholic acid			1.0	65.7	21.9	18.9	46.8	71.2
76	4	56		» »			0		3.0			
77	4	55		Cholic acid			1.0	53.6	27.3	24.3	29.3	54.7
76	4	56		» »			0		3.0			
55	4	60		Desoxycholic acid			1.0	54.1	32.7	29.1	25.0	46.2
56	4	62		» »			0		3.6			
79	4	58		Dehydrocholic acid			1.0	59.3	34.3	31.9	27.4	46.2
78	4	58		» »			0		2.4			
81a	4	61	7	None	9	0	1.0	62.1	31.7	28.5	33.6	54.1
94	4	98	4	»	9		1.0	98.8	22.6	19.4	79.4	80.3
84a	4	60	7	Cholic acid	9		1.0	60.7	16.8	13.6	47.1	77.6
85a	4	57	7	Desoxycholic acid	9		1.0	59.3	22.8	19.6	39.7	66.9

[1] Oleic acid.

corresponding control group, but the amounts absorbed in the two groups were almost identical. When given in fat-free diet containing 1 per cent cholesterol clearly none of the bile acids tested raised the absorption. As a matter of fact, lower levels of absorption were found in the groups receiving the bile acids than in the control groups fed cholesterol only. It therefore seems justified to conclude that the added bile acids had no effect on the absorption of cholesterol on fat-free diet.

The experiments in which bile acids were given together with arachis oil or oleic acid in the diet, gave less consistent results. The experiment on group 44 which was given cholic acid with 5 per cent arachis oil seems to demonstrate a stimulating effect of cholic acid, in agreement with the findings of FRIEDMAN, BYERS and SHIBATA (1953). However, no definite effect was found in a parallel group or in the groups given desoxycholic or dehydrocholic acid together with 5 per cent arachis oil. Whether cholic and desoxycholic acid stimulated the absorption of cholesterol when given together with 9 per cent oleic acid can not be stated with certainty because of the variable results obtained in the control groups.

Deposition. The effect of the bile acid ingestion on the concurrent deposition of cholesterol in the liver and on the serum cholesterol level is seen in table 2. In all groups fed cholesterol a significant cholesterol deposition which was further increased by bile acids, was observed. Since no distinct increase in the liver cholesterol content was found in the rats fed bile acids in the absence of cholesterol, the extra cholesterol deposited when both cholesterol and bile acids were fed must have been — at least predominantly — of exogenous origin.[1] The effect on the deposition was pronounced with cholic and desoxycholic acid, while in comparison dehydrocholic had only a moderate effect. These findings are in principal agreement with previous reports (LOEFFLER 1928, HUMMEL 1929).

The observations on the effect of the bile acids on the serum cholesterol levels were likewise in qualitative agreement with observations of previous investigators, in so far as the groups given bile acids in most cases showed higher serum cholesterol values than those fed cholesterol only. However, the variability in the

[1] Any effect of the bile acids on the deposition of endogenous cholesterol would be eliminated by the method of calculation (see table 2).

The effect of bile acid ingestion on cholesterol deposition in the liver and on the serum cholesterol.

Group no.	Feeding period.[1] Days	Additions to basal diet: Bile acid	Additions to basal diet: Fat %	Additions to basal diet: Cholesterol %	Liver wt. g	Cholesterol in liver: Free %	Cholesterol in liver: Chol. esters[2] %	Cholesterol in liver: Total %	Cholesterol in liver: Total mg	Cholesterol in liver: Deposition mg	Serum cholesterol: Total mg per 100 ml	Serum cholesterol: Free mg per 100 ml
41	21	None	0	0.25	4.10	0.27	0.79	0.74	30	15	61 ± 7[3]	—
28		»		0	4.99	0.20	0.16	0.30	15		—	—
39		Cholic acid		0.25	4.48	0.30	3.17	2.19	98	81	146 ± 35	—
40		» »		0	4.12	0.21	0.34	0.41	17		56 ± 6	—
53	11	None	0	1.00	2.66	0.30	1.98	1.48	39	24	139 ± 7	—
28		»		0	4.99	0.20	0.16	0.30	15		—	—
43		Cholic acid		1.00	3.55	0.34	3.61	2.49	88	71	164 ± 10	27.7 ± 5.1
40		» »		0	4.12	0.21	0.34	0.41	17		56 ± 6	—
45		Desoxycholic acid		1.00	3.41	0.38	3.93	2.72	93	80	214 ± 29	31.7 ± 9.5
54		» »		0	3.07	0.28	0.22	0.41	13		126 ± 19	—
47		Dehydrocholic acid		1.00	2.74	0.34	2.64	1.91	52	35	111 ± 18	20.7 ± 3.2
48		» »		0	2.64	0.29	0.60	0.65	17		63 ± 10	
52	14	None	5	1.00	3.46	0.33	3.46	2.39	83	71	123 ± 10	17.3 ± 3.5
51		»		0	2.35	0.29	0.34	0.49	12		79 ± 8	—
75		None		1.00	2.88	—	—	2.96	85	79	136	30.6
74		»		0	2.88	—	—	0.21	6		84	18.2
44		Cholic acid		1.00	3.92	0.56	8.58	5.67	222	211	199 ± 29	29.7 ± 5.7
76		» »		0	2.64	—	—	0.42	11		68	
77		Cholic acid		1.00	3.38	—	—	4.80	162	151	252	48.1
76		» »		0	2.64	—	—	0.42	11		68	11.5
55		Desoxycholic acid		1.00	4.06	0.54	6.12	4.18	170	148	87 ± 12	—
56		» »		0	3.83	0.25	0.55	0.58	22		83 ± 18	—
79		Dehydrocholic acid		1.00	3.17	—	—	3.64	115	107	165	39.3
78		» »		0	2.58	—	—	0.30	8		65	8.7

[1] The animals were given the experimental diets for 3—4 days before the absorption measurements were started.
[2] (Total cholesterol — free cholesterol) × 1.68.
[3] Standard error of the mean.

Table 3.

Summary of the effect of bile acid ingestion on the deposition of cholesterol in the liver.

Additions to basal diet			Cholesterol absorption per day mg	Cholesterol deposition in liver per day mg	Deposition in per cent of absorption
Bile acid	Fat %	Cholesterol %			
None	0	0.25	15.1	0.7	4.6
Cholic acid			15.2	3.9	25.5
None	0	1.0	28.8	2.2	7.6
Cholic acid			24.4	6.5	26.6
Desoxycholic acid			21.9	7.3	33.3
Dehydrocholic acid			22.2	3.2	14.4
None	5	1.0	29.6	5.1	17.2
»			24.7	5.6	22.7
Cholic acid			46.8	15.1	32.3
» »			29.3	10.8	36.9
Desoxycholic acid			25.0	10.6	42.4
Dehydrocholic acid			27.4	7.6	27.7

serum cholesterol values was considerable, and in certain cases no correlation between liver and serum cholesterol levels was found.

The data demonstrate clearly that when increased levels of cholesterol were present in liver and blood, the main increase occurred in the esterified cholesterol fraction.

The present finding that dietary bile acids markedly increased the deposition of cholesterol in rats where the absorption was not increased indicates a post-absorptive effect of the bile acids. Table 3 is instructive in this respect. In the groups where cholic and desoxycholic acid were added, the fraction of absorbed cholesterol being deposited was considerably increased. This effect of the bile acids is particularly evident in the series of animals on the fat-free diet. However, even in the one experiment where the addition of cholic acid appeared to have increased the absorption (group 44), this extra effect on the deposition is evident.

The deposition of cholesterol, in per cent of the absorbed amount, was in all cases greater on the fat containing than on the fat-free diet. This result suggests that dietary fat influences the fate of absorbed cholesterol, in agreement with previous findings (PIHL 1955).

Table 4.

Table 4.

The effect of cholic acid ingestion on cholesterol synthesis in liver slices.

Experiment no.	Rat no.	Body wt. g	Regimen			Days fed	Liver		Per cent of added C^{14} recovered in		
			Diet	Cholesterol %	Cholic acid %		g	Cholesterol %	Cholesterol	Fatty acids	CO_2
1	384	78	Stock diet	0	0	13	2.89	0.24	9.65	0.34	46.7
	385	80	» »	0	0	13	2.51		3.53	0.13	46.1
	386	76	» »	0	0	13	4.00		6.60	0.64	48.0
	383	98	» »	1	0	13	4.59	1.91	0.02	0.42	59.0
	380	104	» »	1	0	13	4.36		0.01	0.30	53.7
	381	80	» »	1	0	13	3.51		0.05	0.20	49.0
2	399	69	Stock diet	0	0.5	8	3.07	0.62	0.80	0.80	56.7
	400	63	» »	0	0.5	8	2.55		1.15	0.32	57.9
	401	64	» »	0	0.5	8	2.61		0.99	0.93	62.0
	403	76	» »	1	0.5	8	3.65	3.90	0.02	0.29	50.2
	402	55	» »	1	0.5	8	3.31		0.04	0.44	51.0
	404	70	» »	1	0.5	8	3.83		0.01	0.95	45.9
3	389	178 [1]	Stock diet	0	0.5	8	9.48	[2]	1.39	0.17	46.3
	393	185 [1]	» »	0	0	8	7.38		0.98	0.25	47.7
	392	132 [1]	» »	0	0.5	8	5.86		0.20	0.15	44.2
	395	145 [1]	» »	0	0	8	6.24		3.24	0.22	43.3

[1] Pair-fed.

[2] The average cholesterol content of the liver was 0.54 per cent and 0.25 per cent for the rats given cholic acid and no cholic acid respectively.

Synthesis. It was argued above that the extra cholesterol deposited in the rats fed cholesterol and bile acids was predominantly of exogenous origin. However, an effect of the bile acids on the synthesis might have contributed to the net result. In order to test this possibility the effect of cholic acid feeding on the synthesis of cholesterol was studied in surviving liver slices (table 4).

Following the ingestion of 1 per cent cholesterol for 13 days (experiment 1) the incorporation of labelled acetate into cholesterol was almost abolished in agreement with previous reports (TOMKINS, SHEPPARD and CHAIKOFF 1953, LANGDON and BLOCH 1953). The incorporation of acetate carbon into fatty acids and respiratory CO_2 was not affected. When 0.5 per cent cholic acid was added to the diet (experiment 2) the typical effect on the deposition was observed. In the groups fed cholesterol, the cholesterol synthesis was again almost abolished. It is therefore clear that an effect of the cholic acid on the synthesis did not contribute to the increased cholesterol deposition.

In the livers of the rats fed cholic acid without cholesterol (experiment 2) the incorporation of acetate into cholesterol was definitely lower than in the rats fed stock diet only (experiment 1). In experiment 3 the synthesis was compared in pair-fed animals given stock diet with and without the addition of cholic acid. Clearly the results in this experiment were not conclusive. However, considered in their entirety the data suggest that cholic acid ingestion may inhibit the hepatic synthesis of cholesterol.

The fact that in the experiments here presented, increased deposition of cholesterol was observed under conditions where neither the absorption nor the hepatic synthesis was increased, indicates that the extra deposition was due to a reduced catabolism of cholesterol in the liver.

Discussion.

The main conclusion emerging from the present study is that administration of bile acids may reduce the capacity of rats to catabolize absorbed cholesterol. This interpretation is consistent with the previously mentioned findings (HUMMEL 1929) that ingestion of bile acids, in the absence of dietary cholesterol, increased the liver cholesterol content of normal mice and prevented the disappearance of cholesterol previously stored in the liver.

The observed effect of the bile acids on the metabolism of cholesterol in the liver is possibly associated with the well-known fact that cholesterol is degraded to bile acids in this organ (BLOCH, BERG and RITTENBERG 1943, BERGSTRÖM 1952, SIPERSTEIN and CHAIKOFF 1952). Since the enterohepatic circulation is known to operate with remarkable efficiency (SIPERSTEIN, HERNANDEZ and CHAIKOFF 1952) the livers of animals consuming bile acids will presumably be flooded with the products of cholesterol degradation. Cholic acid is known to be the main end product of the hepatic cholesterol degradation in the rat (BYERS and BIGGS 1952, BERGSTRÖM 1952) and evidence has been presented that desoxycholic acid is an intermediate in its formation (BERGSTRÖM, ROTTENBERG and SJÖVALL 1953) while on the other hand dehydrocholic acid does not appear to be formed from cholesterol in the liver. The quantitatively different effects of the bile acids observed in the present and previous studies may possibly be related to these facts. SWELL, FIELD and TREADWELL (1953) have recently observed that the relative ability of various bile acids to activate pancreatic cholesterol esterase *in vitro* paralleled their ability to increase the blood cholesterol level of rats in feeding experiments (SWELL, FLICK, FIELD and TREADWELL 1953). The inference that the *in vivo* results could be accounted for by the relative effects of the bile acids on pancreatic cholesterol esterase in the intestine is, however, not supported by the present data.

In numerous investigations bile acids have been included in the experimental diets with the purpose of increasing cholesterol absorption. However, the present results indicate that a significant part of the effect of bile acids on blood and tissue cholesterol concentrations observed in prolonged feeding experiments may be due to a metabolic effect of the bile acids.

Summary.

The effect of cholic, desoxycholic and dehydrocholic acid on the intestinal cholesterol absorption and on the simultaneous deposition of cholesterol in the liver has been studied in balance experiments on rats.

Bile acid ingestion on a fat-free diet did not increase the absorption of cholesterol.

When the effect of the bile acids were tested on a diet containing 5 per cent arachis oil or 9 per cent oleic acid, a stimulating effect

on cholesterol absorption was only observed in one group fed cholic acid with arachis oil. The variability in these experiments was considerable and no clearcut conclusion could be drawn concerning the effect of bile acids on the intestinal absorption of cholesterol in the presence of dietary fat or fatty acids.

The deposition of cholesterol in the liver was markedly increased by cholic or desoxycholic acid ingestion and moderately but definitely increased by dehydrocholic acid ingestion.

The serum cholesterol levels of the rats fed bile acids reflected in most cases the increased deposition in the liver.

The simultaneous measurements of cholesterol absorption and deposition indicated a post-absorptive effect of dietary bile acids on cholesterol metabolism.

Cholic acid feeding, in the presence or absence of dietary cholesterol did not increase the hepatic cholesterol synthesis *in vitro*, as judged by the incorporation of 1-C^{14} acetate into cholesterol in surviving liver slices.

The inference has been made that bile acid ingestion probably interferes with the catabolism of cholesterol in the liver.

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From the Department of Pharmacology and Institute of Physiology, University of Lund, Sweden.

The Mode of Neuromuscular Block Caused by Acetylcholine, Nicotine, Decamethonium and Succinylcholine.[1]

By

STEPHEN THESLEFF.

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Neuromuscular blocking agents may be divided into those which prevent depolarization of the motor end-plate region and into those which by themselves depolarize this region. The nature of the neuromuscular block caused by the first group, *i. e.* tubocurarine-like agents, is fairly well understood. It is generally accepted that they block the transmission by decreasing the sensitivity of the end-plate region to the transmittor substance, and that they do not alter the membrane potential.

The mechanism of the neuromuscular block caused by the second group, *i. e.* agents which by themselves depolarize the end-plate region, is less well known. Of the agents belonging to this group, the effects of acetylcholine, nicotine, decamethonium and succinylcholine have been most studied. The investigations of BROWN, DALE and FELDBERG (1936) and of BACQ and BROWN (1937) showed that acetylcholine and nicotine may cause a neuromuscular block, and BUCHTHAL and LINDHARD (1937) and KUFFLER (1943) demonstrated that these agents specifically depolarize the end-plate regions of skeletal muscle. BARLOW and ING (1948) and PATON and ZAIMIS (1949) introduced decametho-

[1] A preliminary report on the results obtained with acetylcholine has been published (THESLEFF 1955).

nium as a neuromuscular blocking substance, and BURNS and PATON (1951) investigated its effect on the gracilis muscle of the cat. Using surface electrodes, BURNS and PATON were able to demonstrate that the neuromuscular block produced by decamethonium and acetylcholine was well correlated to the degree of depolarization caused by these agents at the end-plate regions of the muscle, as judged by demarcation-potential measurements. On the basis of their findings, they suggested that both decamethonium and acetylcholine interrupt neuromuscular transmission by depolarizing persistently the end-plate region. This theory of the mode of action has found wide acceptance, and has been adopted to explain the neuromuscular block caused by succinylcholine (GINZEL et al. 1951 A, THESLEFF 1952).

As long as no studies have been made of the direct relation of such a neuromuscular block to the membrane potential of single end-plates, the aforementioned mode of action must, however, be regarded as hypothetical. Consequently, the immediate purpose of the present investigation was a study of the effect of acetylcholine, nicotine, decamethonium and succinylcholine on the end-plate potential and resting membrane potential of single muscle fibres, and thereby of the direct relationship between the neuromuscular block and the membrane depolarization caused by these agents.

Methods.

The experiments were performed on the sartorius nerve-muscle preparation of the frog (Rana temporaria) during October to January, at a room temperature of about 21° C.

External recording. For recording of the demarcation potential, the muscles were mounted vertically in a holder with their pelvic end uppermost. The nerve was lifted out of the Ringer's fluid and stimulated with single supramaximal shocks at a frequency of 40/min. The recording was made when the fluid was run out at an approximately constant rate, with one electrode in the upper end of the muscle and the other electrode in the bath. The potential distribution along the muscle surface was recorded continuously on a slowly moving film. The muscle had to be prepared with care to avoid injury. To diminish injury potentials, the muscle was stored for 24 hours at 4° C. Muscles which showed potential differences greater than 1 mV were discarded. A conventional battery-operated dc-amplifier with a frequency response flat to 10,000 was used.

Intracellular recording. The recording was made with capillary microelectrodes with an external tip diameter of less than 0.5 μ, filled with 3 M—KCl (for details of the technique, see NASTUK and HODGKIN

1950 and FATT and KATZ 1951). Only electrodes with a resistance of less than 10MΩ were used, most frequently 1—3 MΩ.

The freshly-dissected muscle was placed on a perspex block through which it was illuminated, and the preparation was viewed with a binocular dissecting microscope. The microelectrode was connected to the input stage through an Ag—AgCl electrode, and the bath contained an agar-Ringer fluid bridge connecting to an Ag—AgCl electrode. The bath electrode led to earth via small resistances, through which a calibration voltage could be applied. The microelectrode assembly was placed on a Zeiss slide micromanipulator.

The end-plate region of single muscle fibres with their point of maximum e. p. p. was located in the curarized muscle (d-tubocurarine chloride $4—5 \times 10^{-6}$) as described by FATT and KATZ. By careful mapping of the microscopic field, it was possible to identify the fibre and to return to the same spot within 50 μ. To remove the curare solution, the muscle was washed for 60 minutes with Ringer's fluid. During the experiment, the Ringer's fluid in the bath was changed about every 30 minutes.

For recording, a dc-amplifier was used (frequency response flat 0--35,000) with a cathode follower (RCA 954) as input stage, as described by NASTUK and HODGKIN. The grid current was $< 5 \times 10^{-11}$. E. p. p. s. were recorded with single sweep, and changes in membrane potential on a slowly moving film.

Measurement of the electrical threshold of the muscle membrane. The determinations were made as described by CASTILLO and ENGBAEK (1954). Two microelectrodes were inserted less than 50 μ apart in the nerve-free pelvic part of a muscle fibre. One of the electrodes was connected to a circuit containing a source of E. M. F. delivering rectangular pulses of current. The second microelectrode was used to record the resulting potential changes. The largest potential change which just failed to flare up into an action potential was taken as a measure of "threshold".

Solutions. The Ringer's fluid had the following ionic composition expressed in m.mole/l.: Na 115; K 2.1; Ca 1.8; all the salts were used as chlorides. When the effects of acetylcholine were tested, the cholinesterase activity of the muscle was inhibited by the addition to the bath of neostigmine bromide 10^{-6}, 20 minutes prior to the addition of acetylcholine. The compounds used in the study were: d-tubocurarine chloride (Abbott), acetylcholine iodide (Hoffmann La-Roche), nicotine alkaloid (Eastman Kodak Company), decamethonium iodide (Kabi), succinylcholine iodide (Vitrum), choline chloride (Pharmacia) and neostigmine bromide (Leo). Fresh solutions of the compounds were prepared for each day's experiment. Solutions of acetylcholine, nicotine and succinylcholine were prepared immediately prior to use. Unless otherwise stated, the concentrations given are expressed in gm/ml.

Inhibition of cholinesterase activity. The inhibition of cholinesterase activity (I 50) in the sartorius muscle by decamethonium iodide and neostigmine bromide was determined according to the electrometric method of TAMMELIN (1953).

Results.

Effect of acetylcholine and nicotine on the demarcation potential of the muscle.

With external recording, a total of 16 experiments were made; 6 experiments with acetylcholine and 10 with nicotine.

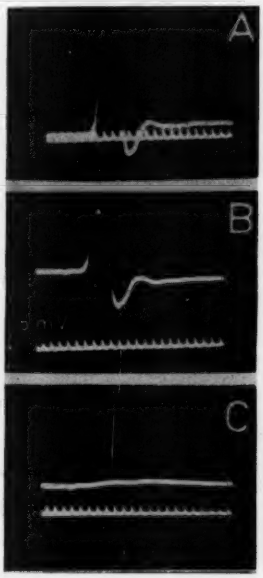

Fig. 1. A. The action potential at the end-plate region of normal muscle as recorded with extracellular electrodes. B. The action potential in the same region of the muscle during maximum depolarization caused by acetylcholine iodide 2×10^{-5}. C. Five minutes later, the depolarization of the end-plate region has almost subsided and neuromuscular transmission is completely blocked. Time 5 msec.

In the presence of neostigmine bromide $1—2 \times 10^{-6}$, acetylcholine iodide in a concentration of $1—2 \times 10^{-5}$ caused a neuromuscular block which was complete 6—15 minutes after the addition of acetylcholine to the bath. Immediately after the addition of the substance, a maximum negativity of the muscle surface was observed at points corresponding to the end-plate regions of the muscle. The recorded negativity was 5—10 mV and it started to subside within a few minutes. During this phase of most marked negativity, stimulation of the motor nerve elicited muscle twitches, and the action potentials recorded were only moderately reduced in size (Fig. 1). When the negativity of the muscle end-plate region started to subside, the action potentials were reduced, and a complete neuromuscular block developed at

about the same time as the demarcation membrane potential reached its normal value (Figs. 1 and 2). A further addition of acetylcholine to the muscle bath no longer altered the demarcation potential of the muscle. Neuromuscular transmission was not restored until the fluid in the bath was changed and the muscle washed with fresh Ringer's fluid for 15—45 minutes.

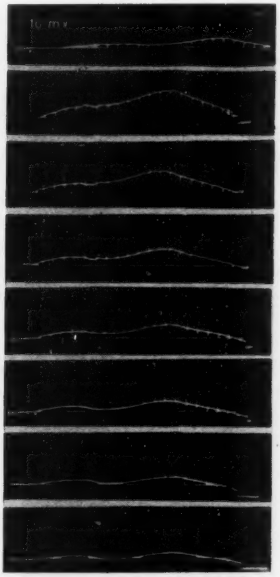

Fig. 2. The uppermost curve shows the demarcation and action potentials of normal muscle soaked in Ringer's fluid and neostigmine bromide 10^{-6}. The subsequent curves are obtained at one minute intervals after the addition to the muscle bath of acetylcholine iodide 2×10^{-5}.

The effect of nicotine in a concentration of $1—2 \times 10^{-5}$ was similar to that observed with acetylcholine. The only exception was that a complete neuromuscular block developed earlier with nicotine than with acetylcholine, and at a time when the negativity of the muscle surface had not completely subsided. After nicotine, it was more difficult to restore neuromuscular transmission by washing with Ringer's fluid than after acetylcholine.

The disappearance of the negativity of the muscle surface might be due to a spread of depolarization along the whole muscle. That this is unlikely was shown by recording the spread of negativity after a crush injury to the muscle. The injury potential reached a value of about 18 mV and spread along the muscle to the extent of only 1 mm in one hour (Fig. 3). Subsequent experiments with intra-

cellular recording provided another explanation of the disappearance of depolarization.

Neuromuscular block and resting membrane potential of single muscle fibres.

The experiments were made with capillary microelectrodes on a total of 22 muscles, in which 73 end-plates and their point of maximum e. p. p. were located. The mean resting membrane potential of the end-plates was 92.6± 1.05 mV (S. E. of mean).

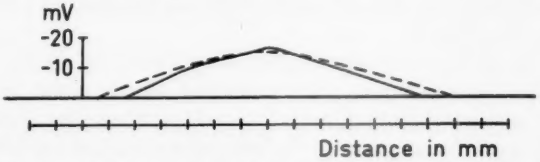

Fig. 3. The spread of injury potential along the muscle surface. The solid line shows the distribution of injury potential 3 minutes after a crush injury to the muscle, and the dotted line the same potential 60 minutes later.

The effects of acetylcholine iodide were investigated in 10 muscles (22 end-plates), of nicotine in 5 muscles (23 end-plates), of decamethonium iodide in 4 muscles (15 end-plates) and of succinylcholine iodide in 3 muscles (13 end-plates).

In the presence of neostigmine bromide 10^{-6}, acetylcholine iodide in a concentration of $0.5—2 \times 10^{-5}$ produced a neuromuscular block. The addition of acetylcholine to the muscle bath led to fibrillar twitches of the muscle fibres. When these twitches had subsided after about 60 seconds, the tip of the microelectrode was reinserted in a previously located end-plate region of a single muscle fibre and the resting membrane potential continuously recorded. These concentrations of acetylcholine brought about a considerable reduction in the membrane potential at the end-plate region. The depolarization nevertheless subsided spontaneously without removal of the acetylcholine, and the membrane potential was restored to about its original value within 7—15 minutes, as shown in Fig. 4 A. A subsequent addition of acetylcholine to the bath did not significantly change the membrane potential (Fig. 4 A). The same results were obtained if the microelectrode, instead of being continuously inserted at the end-plate region, was withdrawn and inserted at short intervals. During the period of depolarization, a neuromuscular block developed, and persisted despite repolarization of the membrane to the normal level. End-plate

potentials could sometimes be recorded during the phase of depolarization but never in the later phase when the resting membrane potential was once more normal. During the phase of complete neuromuscular block, the mean resting membrane potential of 22 end-plates was 88.7 ± 1.17 mV.

Neuromuscular transmission remained completely blocked as long as acetylcholine was in the muscle bath and could be restored only by washing the muscle in Ringer's fluid for 15—45 minutes. During the period of washing, the resting membrane potential remained normal, and end-plate potentials reappeared before the block was relieved.

In the absence of neostigmine, a qualitatively similar neuromuscular block was produced by acetylcholine in about ten times higher concentrations. Choline chloride in concentrations corresponding to the amounts of acetylcholine used had no detectable influence on the resting membrane potential or on the neuromuscular transmission.

The results obtained with nicotine, decamethonium and succinylcholine were qualitatively similar to those observed with acetylcholine. A neuromuscular block was obtained with the following concentrations: nicotine $1.5 - 3 \times 10^{-5}$, decamethonium iodide $4 - 8 \times 10^{-5}$, and succinylcholine iodide $1 - 2 \times 10^{-5}$. The first effects observed were fibrillar twitches of the muscle fibres and depolarization of the end-plate regions. The depolarization was not fully as marked, nor was the membrane potential restored as rapidly as with acetylcholine (Fig. 4). After nicotine in particular, the depolarization subsided slowly and the resting membrane potential was not completely restored (Fig. 4 B). When the depolarization had subsided, a subsequent addition of any of the compounds or of acetylcholine no longer changed the membrane potential. The changes observed in the resting membrane potential were invariably seen only at the end-plate region and never in the nerve-free part of the muscle fibre.

As with acetylcholine, the neuromuscular block developed during the phase of depolarization and persisted when the membrane was repolarized. The mean resting membrane potential of end-plate regions during a complete neuromuscular block was with nicotine 83.3 ± 1.44 mV (23 measurements), with decamethonium 89.8 ± 2.36 mV (15 measurements) and with succinylcholine 87.1 ± 2.92 mV (13 measurements). Neuromuscular transmission was completely blocked as long as the compounds remained in the muscle

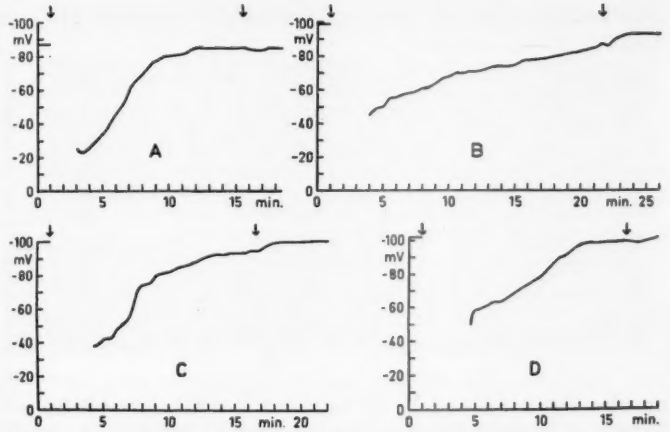

Fig. 4. Continuous recording of the resting membrane potential at the end-plate region of single muscle fibres. At the arrows, the following agents are added to the Ringer's fluid: A. acetylcholine iodide 10^{-5}, B. nicotine 1.5×10^{-5}, C. decamethonium iodide 4×10^{-5}, D. succinylcholine iodide 2×10^{-5}.

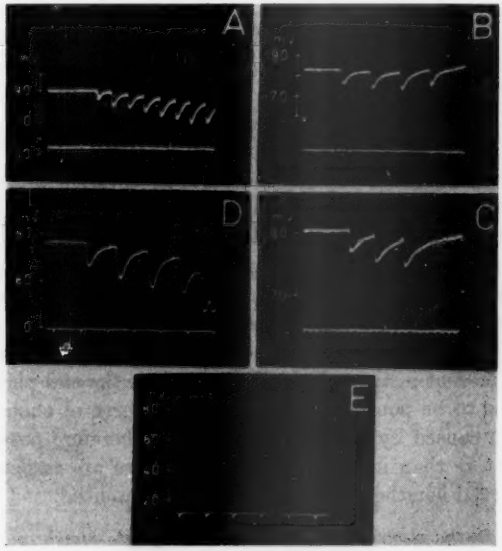

Fig. 5. Effect of repetitive stimulation of the motor nerve on end-plate potentials when neuromuscular transmission is blocked by: A. acetylcholine iodide 2×10^{-5}, B. nicotine 1.5×10^{-5}, C. decamethonium iodide 8×10^{-5}, D. succinylcholine iodide 1.5×10^{-5}, E. d-tubocurarine chloride 5×10^{-6}. Time 5 msec.

bath and was restored only by washing the muscle in Ringer's fluid for 15—45 minutes.

Effects on the end-plate potential.

The end-plate potentials observed during a neuromuscular block caused by acetylcholine, nicotine, decamethonium and succinylcholine were increased in size by repetitive stimulation of the motor nerve in the same characteristic manner as during a block caused by d-tubocurarine (Fig. 5).

The time-course of an end-plate potential during neuromuscular block caused by acetylcholine, nicotine and succinylcholine was similar to that observed with d-tubocurarine. With decamethonium, the end-plate potential had a longer duration and resembled that observed after the addition of neostigmine to a curarized muscle (Fig. 6 III and I). ECCLES and MACFARLANE (1949) and FATT and KATZ (1951) have shown that neostigmine markedly prolongs the duration of an end-plate potential and that this prolongation is presumably due to the anticholinesterase activity of neostigmine. Decamethonium is not known to be a potent inhibitor of cholinesterases, and it was therefore of interest to determine its potency as an inhibitor of cholinesterases in the sartorius muscle of the frog. Such a determination showed that decamethonium iodide in a concentration of 8×10^{-5} produced a 50 per cent inhibition of the acetylcholinesterases in the muscle. The corresponding concentration of neostigmine bromide was 2.1×10^{-7}. The effect on the end-plate potential of these equipotent concentrations of decamethonium and neostigmine is shown in Fig. 6 III and II. It is evident that decamethonium in this concentration caused a marked prolongation of the end-plate potential, whereas neostigmine in a corresponding concentration had no significant effect. Neostigmine bromide in a concentration of 10^{-6}, as used in Fig. 6 I, caused complete inhibition of the cholinesterase present in the muscle. This effect on the end-plate potential seems to be poorly correlated to the degree of cholinesterase inhibition caused by decamethonium. It is therefore possible that other factors than inhibition of cholinesterase are responsible for the observed lengthening of the end-plate potential.

Effect on the electrical threshold of the muscle membrane.

A decrease in the direct excitability of mammalian muscles treated with acetylcholine has been observed by BROWN (1937) and in that of muscles treated with decamethonium, succinylcho-

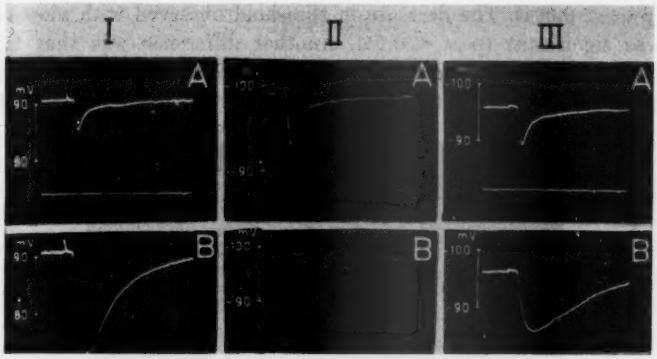

Fig. 6. Effects on the time-course of the end-plate potential of neostigmine and decamethonium. IA, IIA and IIIA show e. p. p.s during neuromuscular block caused by d-tubocurarine chloride 5—10 × 10^{-6}. In IB is seen the effect on these end-plates of neostigmine bromide in a concentration of 10^{-6}, and in IIB its effect in a concentration of 2.1×10^{-7}. IIIB shows the effect of decamethonium iodide in a concentration of 8×10^{-5}. Time 5 msec.

line and nicotine by GINZEL et al. (1951 B and C). The most probable explanation of such a decrease would be an action of these substances on the electrical threshold of the muscle membrane. In order to obtain quantitative data on this action, the electrical threshold of the muscle membrane was determined in muscles treated with these substances. The results are recorded in Table 1.

The elevation in threshold that was observed in muscles treated with decamethonium was highly significant as compared to normal

Table 1.

Compound	Concentration	No. of muscles	No. of fibres	Mean membrane potential in mV	Mean threshold in mV
Normal Ringer's fluid	—	6	86	82.4 ± 0.82[1]	31.4 ± 0.88[1]
Neostigmine bromide + acetylcholine iodide	$10^{-6} + 2 \times 10^{-5}$	3	50	80.4 ± 0.74	33.4 ± 0.41
Nicotine	1.5×10^{-5}	4	66	77.2 ± 0.62	28.1 ± 1.02
Decamethonium iodide	8×10^{-5}	4	59	77.4 ± 0.90	37.0 ± 1.28
Succinylcholine iodide	2×10^{-5}	5	82	80.4 ± 0.77	30.8 ± 1.17

[1] S. E. of mean.

($p = < 0.001$). The decrease in threshold observed with nicotine was significant ($p = < 0.05$). Another difference was that the slope of the depolarization potential was much steeper in muscles treated with decamethonium than in normal muscles (Fig. 7).

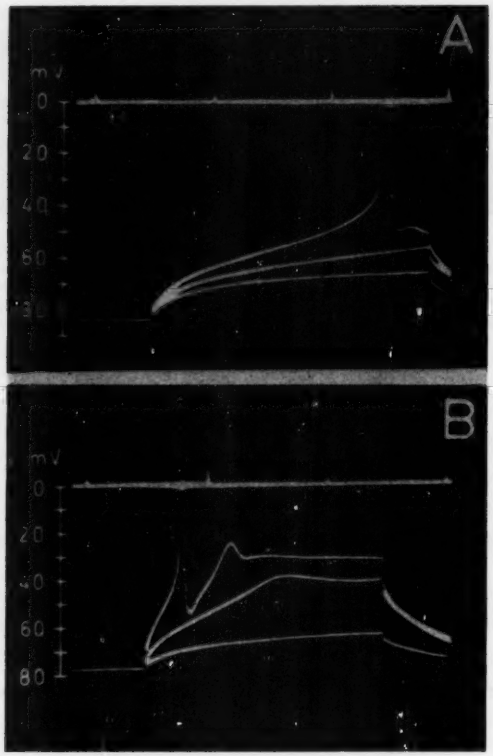

Fig. 7. Effect of decamethonium on the electrical threshold of the muscle fibre membrane. A. muscle soaked in normal Ringer's fluid. B. muscle soaked in solution containing decamethonium iodide in a concentration of 8×10^{-5}. Time 20 msec.

These results differ from those of BROWN and of GINZEL et al., using direct electrical stimulation of the whole muscle. The discrepancy may be due to specie variations but is most probably to be ascribed to the difficulty of comparing quantitative measurements with qualitative observations.

Discussion.

The present investigation has shown that acetylcholine, nicotine, decamethonium and succinylcholine have a qualitatively similar action on the neuromuscular transmission in the frog. In appropriate concentrations, they cause an initial depolarization of the end-plate regions of the muscle and a neuromuscular block. The depolarization of the end-plate regions is of short duration, and the membrane potential is spontaneously restored to about its normal value without removal of the agent. The neuromuscular block develops during the period of depolarization and persists despite repolarization of the muscle membrane.

A closer study of the relationship between membrane potential and neuromuscular block reveals that the period of depolarization is not accompanied by a maximum of neuromuscular block. Moreover, a complete neuromuscular block does not develop until the membrane potentials of the end-plate regions are restored to about their normal values (Figs. 1 and 2). Thus, it is not possible to show any relationship between the membrane depolarization and the neuromuscular block caused by the agents investigated. Although the depolarization of the end-plate regions may nevertheless be responsible to some extent for the initial neuromuscular block, it must be emphasized that this can apply only during the very first minutes and that, subsequently, the neuromuscular block is not associated with any depolarization of the muscle membrane. The threshold depolarization for causing a neuromuscular block occurs at a membrane potential of 57—52 mV, as demonstrated by JENERICK and GERARD (1953).

During the block of neuromuscular transmission, application of acetylcholine to the muscle no longer elicits any depolarization of the muscle membrane, and the end-plate potentials are found to have disappeared. Furthermore, a presynaptic potentiation is observed similar to that described with d-tubocurarine by ECCLES, KATZ and KUFFLER (1941). These findings indicate that the end-plates do not react to the depolarizing effect of acetylcholine.

It can therefore be concluded that the neuromuscular block caused by acetylcholine, nicotine, decamethonium and succinylcholine in the frog is not due to a persistent depolarization of the end-plate regions of the muscle or of adjacent muscle membrane, but to a decrease in sensitivity of the end-plates to the transmittor substance.

The finding of BUCHTHAL and LINDHARD (1939) that the end-

plate region of lizard's striated muscle fibre is refractory to a second dose of acetylcholine, as well as the statements of FATT (1950 and 1954) that in frog muscle an excess of acetylcholine will render the end-plates unreactive, and that the depolarization caused by acetylcholine is not maintained, are in good conformity with the present results and with the conclusions drawn from them.

There is no evidence that the mode of neuromuscular block caused in the frog by acetylcholine, nicotine, decamethonium and succinylcholine is the same as that produced by these agents at the mammalian neuromuscular junction. If, however, the mode of action were supposed to be the same, an explanation would be provided of many observations that are not in agreement with BURNS and PATON's theory of a persistent depolarization block. These are the findings of ZAIMIS (1952) and of THESLEFF and UNNA (1954) indicating that decamethonium and homologues of decamethonium have both a "tubocurarine-like" and a depolarizing action on certain mammalian and avian muscles. Nicotine has also been reported to have such a "dual" mode of action on the gastrocnemius and tibialis muscle of the cat (BACQ and BROWN 1937). The observations of BURNS and PATON that successive doses of decamethonium produced a progressively smaller depolarization in the gracilis muscle of the cat are also compatible with such a mode of action.

Summary.

The neuromuscular block produced in frog muscle by acetylcholine, nicotine, decamethonium and succinylcholine has been investigated with extracellular and intracellular recording electrodes.

The agents investigated are found to act on the neuromuscular junction in a qualitatively similar way.

They cause a brief depolarization of the end-plate regions, which nevertheless subsides spontaneously without removal of the agents, and restoration of the membrane potential to about its normal value takes place.

A neuromuscular block develops during the phase of depolarization and persists despite repolarization of the muscle membrane.

The period of depolarization is not accompanied by a maximum of neuromuscular block, and a complete neuromuscular block does not develop until the membrane potential of the end-plate regions is restored to about its normal value.

During the block of neuromuscular transmission, the end-plate regions of the muscle are insensitive to the depolarizing effect of the transmittor substance.

It is concluded that the neuromuscular block caused by acetylcholine, nicotine, decamethonium and succinylcholine is not due to a persistent depolarization of the end-plate regions or of adjacent muscle membrane, but to a decrease in sensitivity of the end-plate to the transmittor substance.

The effects of the agents on the time-course of the end-plate potential and on the electrical threshold of the muscle membrane have been investigated.

The results and their implications are discussed with reference to existing theories on the mode of neuromuscular block caused by the agents investigated.

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From the Institute of Medical Physiology, University of Copenhagen.

Effects of Insulin and Muscular Exercise upon the Uptake of Hexoses by Muscle Cells.

By

E. J. HUYCKE[1] and P. KRUHØFFER.

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Introduction.

The fact that insulin promotes the uptake of glucose by muscle cells was clearly established by GEMMILL's (1941) studies on the isolated rat diaphragm. The same conclusion had also been reached from experiments on hepatectomized animals (MANN and MAGATH 1923, BURN and DALE 1924) and perfused hind limbs of cats (BEST 1926, NIELSEN 1933).

Studies on the latter preparation (GAMMELTOFT et al. 1944) showed that, by contrast, insulin is without effect upon the fructose uptake. These experiments seemed to indicate then that the uptake of hexoses by muscle cells is limited by factors of marked steric specificity.

The incompleteness of this specificity is indicated however by the recent work of LEVINE and his group (LEVINE et al. 1950, GOLDSTEIN et al. 1953) who found that insulin promoted the distribution of D-galactose (as well as D-xylose and L-arabinose) in the eviscerated, nephrectomized dog, while no such effect was

[1] The work herein reported was carried out while the author held a United States Government grant under P. L. 584, the Fulbright Act. — Present Address: University of Kansas Medical Center, Kansas City, Kansas.

exerted on D-fructose, D-arabinose, D-mannose, etc. These authors drew the conclusion that insulin increases the rate of entrance into muscle cells of only such monohexoses having the same configuration at the 1, 2 and 3 carbons as D-glucose.

WICK and DRURY (1953) claimed to have obtained confirmatory results from similar experiments performed on rabbits. The confirmatory value of their data seems, however, equivocal. It is true that when carrier-free labelled galactose was used, insulin clearly promoted its elimination from the blood. On the other hand, when besides the labelled galactose, carrier galactose in amounts comparable to those used by LEVINE et al., (1 g/kg of body weight) was added, the three hour volume of distribution of galactose was only about 30 per cent even in insulin-treated animals (cf. their figures 1 and 2). This is in marked disagreement with the figure of 65—70 per cent reported by LEVINE et al.

This discrepancy is unfortunate, since a dissimilarity in the rates of uptake of various monohexoses as indicated by LEVINE et al.'s studies must greatly influence our conception of the nature of the uptake of these substances by cells. It was therefore felt that further work on the effect of insulin on D-galactose distribution was desirable, and it was decided to study the problem in the perfused hind limb preparation. This offers the advantages over the eviscerated animal of being less complex (heart, lungs, central nervous system and all known endocrine glands are lacking) and of permitting maintenance of a steadier blood circulation.

It has been long known — especially from experiments on diabetic patients (cf. *e. g.* ERREBO-KNUDSEN 1948) — that muscular exercise may cause a lowering in the blood glucose concentration, and such findings were naturally taken as an indication that the uptake of glucose by muscle cells is enhanced when they are activated. Since the blood glucose concentration is determined by a multitude of factors such experiments on whole subjects are, however, unable to provide information on the quantitative aspects of the question, *i. e.* on the magnitude of the additional glucose uptake and its time-parameter. This problem was therefore studied in the electrically stimulated hind limb preparation. The same approach has also been used to determine the effect of muscular exercise on the uptake of D-fructose and D-galactose.

Methods and Experimental Technique.

Experimental technique.

Hind limb preparations, weighing between 1.1 and 1.7 kg, were obtained from Amytal-anaesthetized cats of body weight generally between 3.5 and 5.5 kg. The preparations were made and the perfusions performed according to the method designed by NIELSEN (1933) and later somewhat modified (LUNDSGAARD et al. 1936). Two etherized cats served as donors for the (somewhat diluted) defibrinated blood used for the perfusion. Haematocrit values ranged from 25—35. All cats had been starved for 36—48 hours.

Perfusions were carried out at mean arterial pressures between 80 and 100 mm Hg. The minute volume was generally around 40 ml/min. immediately after the perfusion was started, but rose to 60—80 ml/min. within some 15—25 min. and then remained essentially unchanged in the resting preparation.

In experiments on the effect of muscular exercise on hexose uptake the preparations were stimulated tetanically (approx. 80 stimuli per sec.) by two induction coils, one for each limb. The secondary coil terminals were connected to pad electrodes placed over the lumbar plexus and needle electrodes inserted below the Achilles' tendons. Stimulation periods lasting approx. 4 seconds alternated with resting periods of similar duration. Contractions were obtained in extensors as well as flexors and adductors, but the dominant response was extension and a spring dynamometer was arranged to be pulled out by this movement. The strength of the stimulation was adjusted so as to raise the rate of oxygen consumption to about 4 times the resting value (*i. e.* from approx. 3 ml O_2/min. to approx. 12 ml/min.) and this level was maintained by keeping a constant deflection on an oximeter perfused by the venous blood returning from the preparation. There was a prompt vasodilatory response to stimulation, and the minute volume had to be increased within some 5 minutes to approx. 150 ml/min. during stimulation in order to maintain unchanged arterial perfusion pressure.

Analytical Methods and Calculations.

Arterial and venous blood was analysed one or two times during each experiment for oxygen and total carbon dioxide contents (VAN SLYKE's manometric method); oxygen consumption was calculated from the arterial-venous difference and the minute volume. Haematocrit determinations were made occasionally throughout the experiments.

All other analyses were made on serum from arterial blood. Blood samples were centrifuged immediately after they had been withdrawn and the serum either stored to next day in a refrigerator or immediately subjected to protein precipitation.

Glucose and fructose were determined on a 1 : 10 $ZnSO_4$/NaOH filtrate prepared *ad modum* SOMOGYI (1930). Reducing power was measured in the filtrate by HAGEDORN and JENSEN's method (1923)

and "fructose" by BOJESEN's diphenylamine method (1952); when both hexoses were present their concentrations were calculated in a way similar to that used by GAMMELTOFT et al. (1944).

Galactose (in the presence of glucose) was determined by its reducing power after glucose had been removed by glucose oxidase. One-half ml of serum was incubated at 38° C for $1^1/_2$—2 hours with 0.5 ml of 0.2 M phosphate buffer, pH 6.0, containing $^1/_3$ mg of glucose oxidase.[1] Eight ml of $ZnSO_4/H_2SO_4$ (12.5 g of $ZnSO_4$, 7 H_2O + 31.5 ml N H_2SO_4 made up to 1 liter) was added, followed by 1 ml 0.75 N NaOH. Aliquots of the supernatant obtained by centrifugation were used for Hagedorn-Jensen determinations of reducing power. The amount of "glucose" read from their table was multiplied by a factor ranging from 1.28 to 1.35 to correct for the lesser reducing power of galactose and a small loss by precipitation. The use of glucose oxidase for removal of glucose is preferable to yeasting, since yeast is known to give off reducing substances.

Glucose was determined in the presence of galactose by an unpublished glucose oxidase method worked out in this laboratory by Dr. E. BOJESEN. Briefly, the procedure was as follows. A 1 : 16 filtrate of serum was prepared by the $Fe_2(SO_4)_3/BaCO_3$ precipitation procedure of STEINER, URBAN and WEST (1932). Six ml of a solution containing approx. 4 μg of 2.6-dichlorophenolindophenol per ml of 0.5 M sodium phosphate buffer at pH 6.0 plus filtrate containing 10—60 μg of glucose were placed into optically matched Thunberg tubes. One mg of glucose oxidase contained in 0.5 ml of phosphate buffer was placed in the bulb of the stopper. The tubes were alternately evacuated to slight boiling and refilled with nitrogen (passed over heated copper) some 10 times with constant shaking in a water bath at room temperature. Upon closure and mixing the density was immediately measured at 600 $\mu\mu$ in a Coleman Universal Spectrophotometer against a neutral glass filter (density about 0.7). For subsequent readings of high absorptions the glass filter was used for zero setting, for low absorptions the density of the filter was read with the samples used for zero setting. The decrease in density observed after 90 minutes standing at room temperature was directly proportional to the amount of glucose present. The amount of galactose present in the samples caused only a negligible decoloration.

Sucrose was determined on the same supernatant as galactose, 1 ml being used for BOJESEN's procedure (1952).

Concentrations of Evans Blue were determined absorptometrically at 610 $\mu\mu$ on serum diluted 1 : 5 with saline. Serum (obtained before the dye was added to the perfusion blood) similarly diluted with plain saline or saline of known dye concentration served for zero setting and standards, respectively.

Urea concentrations were measured by CONWAY's diffusion procedure (1950).

[1] If sucrose is also present in the serum, glucose oxidase must be free of saccharase activity. Preparations meeting this demand were made by Leo Pharmaceuticals, Ltd., Copenhagen and kindly placed at our disposal.

The "volumes of distribution" of galactose and sucrose were calculated by dividing the amounts added to the perfusing blood by the serum concentrations found at each particular time.

Calculations of the volumes of distributions of urea were made by dividing the amount added by a post-addition minus the pre-addition serum concentration.

Extravascular volumes of distribution of galactose and sucrose are taken as their total volumes of distribution minus the serum (Evans Blue) volume. In the case of urea a further subtraction of erythrocyte water (calculated from the haematocrit values) must be made.

The Evans Blue volumes calculated from the serum concentrations determined 10, 20 and 35 minutes after the addition of the dye to the perfusing blood were found almost identical, and they are therefore assumed to represent the serum volume. The values calculated from later determinations of the serum concentration were found to increase slightly in some experiments and decrease in others. Too much significance should, however, not be attributed to these findings, partly because there may have been a loss of dye from the serum, and partly because after hours enough haemolysis has occurred to introduce an error in the determination of the plasma concentration of the dye.

Results.

Fig. 1 shows the results obtained in one of the earlier experiments where the *effect of insulin* addition on *galactose* "distribution" was tested. After a perfusion period of 10 minutes a single dose (2.5 g in 10 ml) of galactose was added to the perfusing blood, followed after two hours by 20 units of crystalline insulin (in $^1/_2$ ml of saline). It is apparent from the concentration/time curve for galactose that there was a tendency for the slope to decrease before the addition of insulin and to increase again shortly after the addition. Correspondingly, in the curve representing "the volume of distribution of galactose" *versus* time an increase in slope is seen shortly after the addition of insulin.

Theoretically, this finding might be attributed to any of the following possibilities: (1) that insulin promotes the entrance of galactose into muscle cells, (2) that an expansion of the extracellular space has taken place in the latter part of the experiment, following the insulin addition, or (3) that in the same period deterioration of the preparation has occurred with increasing cell permeability for galactose, simulating an entrance-promoting effect of insulin.

To exclude the latter two possibilities sucrose was added in later experiments to serve as an indicator for the extracellular

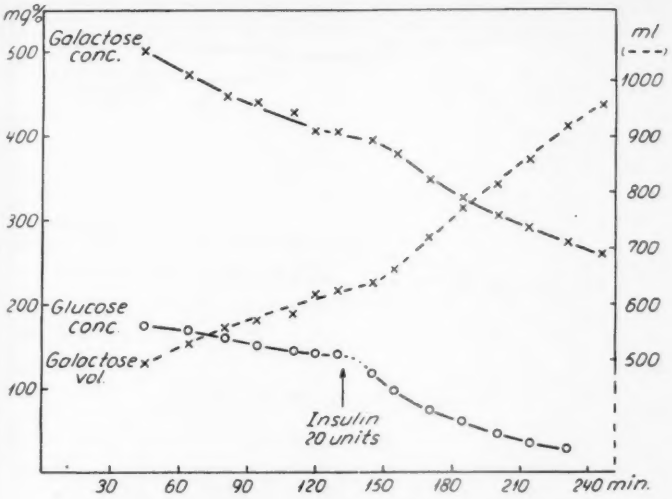

Fig. 1. *Effect of insulin on disappearance of galactose and glucose.*

Weight of perfused hind limb preparation 1,520 g. Start of perfusion at zero time. Single dose of galactose (2.5 g) added to perfusion blood 10 minutes after. Continuous infusion of 3.5 mg of glucose per min. throughout the experiment. 20 units of insulin added at 130 minutes.

Serum glucose and galactose concentrations to be read on left, galactose "distribution volume" on right ordinate.

space, and furthermore control experiments without insulin addition were performed. Sucrose was chosen because it was known from previous experiments (KRUHØFFER 1946) that sucrose has a volume of distribution almost identical with that of inulin and therefore probably only distributes itself in the extracellular space; in addition it has a diffusion coefficient not too different from that of galactose. Furthermore, urea was added to measure total water contents of the preparation + perfusing blood, and Evans Blue to determine the serum volume.

The results from a control experiment are shown in Fig. 2. It is apparent that sucrose is rapidly distributed in a volume larger than the Evans Blue volume. After an hour or so the sucrose volume has nearly reached a maximum value and remains essentially unchanged throughout the rest of the experiment. It also appears that at any time the galactose volume exceeds that of sucrose, and that galactose is continuously lost from the extracellular volume. The rate of this loss is however quite small and,

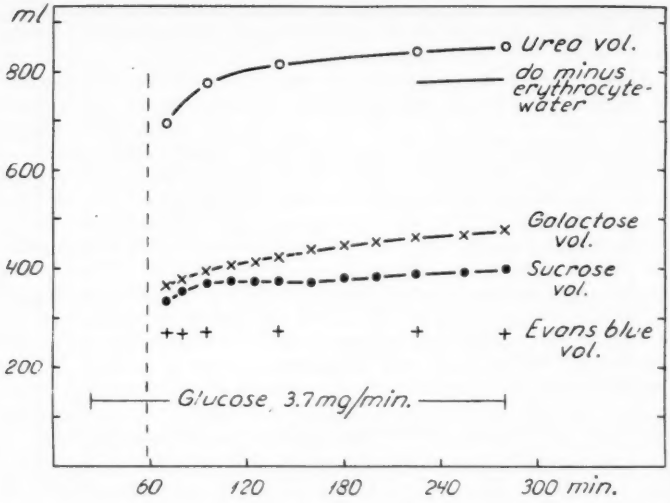

Fig. 2. *Control experiment. "Distribution" of galactose without addition of insulin or electric stimulation.* Distribution volumes for sucrose, urea and Evans Blue determined for comparison.

Weight of hind limb preparation 960 g. Perfusion started at zero time. 1.9 g of galactose *plus* 0.7 g of sucrose *plus* 1.5 g of urea *plus* 16 mg of Evans Blue added at 58 minutes. Continuous addition of glucose as indicated.

clearly, it does not rise towards the end of the perfusion period. Two other control experiments gave similar results.

The results from a typical experiment in which insulin was added appear in Fig. 3. The course of the distribution of sucrose is quite similar to that observed in the control experiments, *i. e.* it is not affected by the addition of insulin. The volume of distribution of galactose initially follows that of sucrose, although at any time it is somewhat higher. Starting about 20 minutes after the addition of insulin it is seen, however, to rise rather steeply, thus deviating from that of sucrose. At the end of the experiment the extravascular volume of distribution of galactose is seen to approach that of the extravascular distribution of urea; in none of three experiments did it, however, reach or exceed the latter.

In one experiment insulin was added at intervals to the perfusion blood before as well as after the addition of galactose + sucrose + urea + Evans Blue. The results are shown in Fig. 4. In this case a deviation between the curves on the volumes of

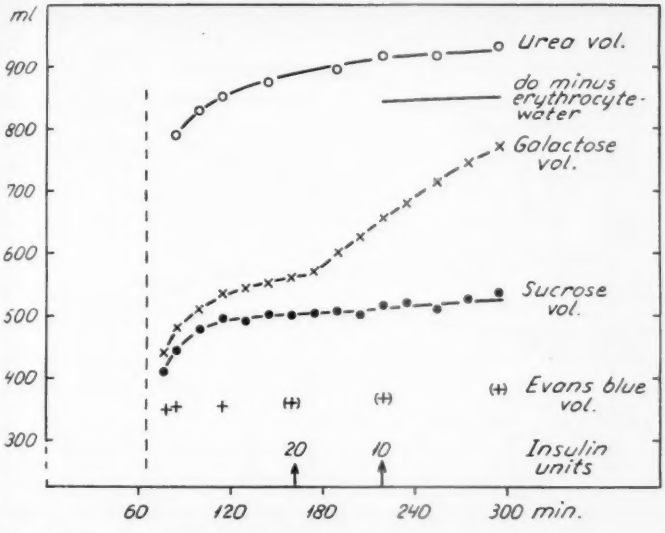

Fig. 3. *Effect of insulin on the "distribution" of galactose;* with distribution volumes for sucrose, urea and Evans Blue determined for comparison.

Weight of hind limb preparation 1,050 g. Start of perfusion at zero time. 2.3 g of galactose *plus* 0.8 g of sucrose *plus* 1.8 g of urea *plus* 20 mg of Evans Blue dissolved in 10 ml added at 65 minutes; addition indicated by vertical broken line. Insulin additions indicated by arrows. Continuous infusion of 3.3 mg of glucose per min. throughout the experiment.

Serum glucose level before insulin addition about 100 mg%.

distribution of galactose and sucrose is apparent shortly after the addition of these substances to the blood. Although the distributions under the action of insulin were observed for over four hours, the extravascular distribution volume for galactose did not reach that of urea.

The effect of *muscular exercise* on distribution of *galactose* was studied in other experiments; the results from a typical experiment are shown in Fig. 5. In the initial period of rest (almost two hours) the volume of distribution of galactose is seen to increase somewhat faster than but rather parallel to the volume of distribution of sucrose. About half an hour after stimulation has begun, the galactose volume increases, however, rather steeply while the sucrose volume remains essentially unchanged. In none of three such experiments did the galactose volume reach the level of the urea volume minus erythrocyte water.

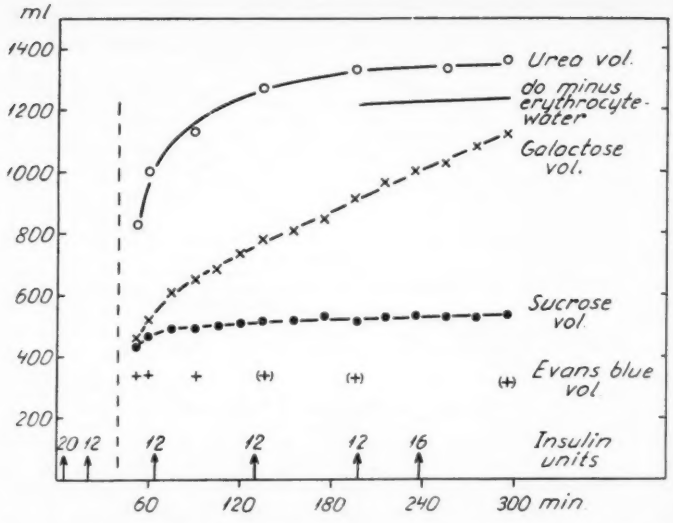

Fig. 4. *Distribution of galactose under continuous influence of insulin;* distribution volumes for sucrose, urea and Evans Blue determined for comparison.

Weight of hind limb preparation 1,625 g. Start of perfusion at zero time. 2 g of galactose *plus* 0.9 g of sucrose *plus* 1.5 g of urea *plus* 20 mg of Evans Blue dissolved in 10 ml added at 40 minutes; addition indicated by vertical broken line. Insulin additions indicated by arrows. Continuous infusion of 7.5 mg of glucose per min. throughout the experiment.

The *effects of muscular exercise* on the uptake of *glucose* and *fructose* appear from Fig. 6, which shows the plasma glucose and fructose concentration in an experiment where a continuous infusion of 4.15 mg glucose/min. and 3.0 mg fructose/min. was maintained during the experiment. After an equilibration period of one hour, electric stimulation was started and maintained for 2 hours.

It is apparent that starting some 20 minutes after the beginning of the stimulation and continuing throughout the stimulatory period a marked drop (280 $\longrightarrow$ 150 mg%) occurs in the glucose concentration. By contrast, the removal of fructose is seen to be unaffected by stimulation, as evidenced by the fact that the slight rise in concentration with time which occurs in the equilibration period remains unchanged during the stimulation period. The slight rise in the fructose concentration level at the start of stimulation is probably due to a shift of water from the extracellular

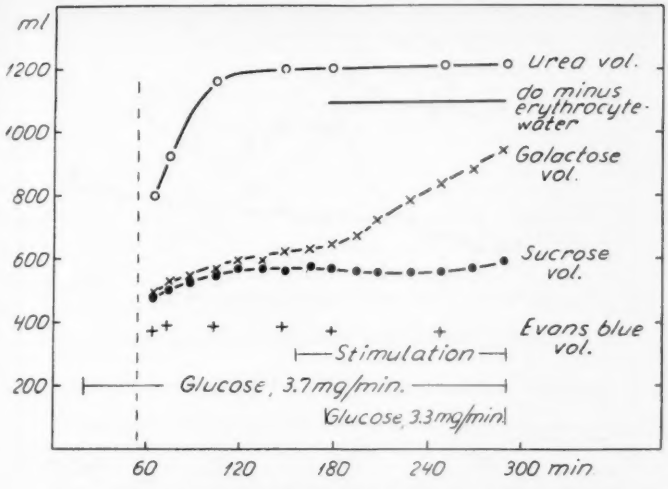

Fig. 5. *Effect of muscular exercise on the "distribution" of galactose;* distribution volumes for sucrose, urea and Evans Blue determined for comparison.

Weight of hind limb preparation 1,350 g. Perfusion started at zero time. 2.4 g of galactose *plus* 0.9 g of sucrose *plus* 1.7 g of urea *plus* 20 mg of Evans Blue added at 54 minutes. Oxygen consumption: 3.4 ml/min. before, and 11.5 ml/min. during stimulation. Continuous additions of glucose as indicated.

into the intracellular space, caused by raised intracellular osmotic pressure. (A slight rise in the haematocrit values of some 1—3 vol.% occurring at stimulation would seem to be another reflection of such a shift.)

While the above experiment gives clear evidence of the enhancing effect of muscle stimulation on glucose uptake, it is not possible to infer the magnitude of the increased rate of uptake from such data. For this reason similar experiments were performed in which, however, a supplementary glucose infusion was started shortly after the stimulation began and maintained to the end of the experiment; the aim was to compensate, as nearly as possible, for the increased rate of glucose uptake.

Figures 7 and 8 show the results obtained in two such experiments. In the experiment of Fig. 7 besides the continuous infusion of 4.6 mg glucose/min. (and 2.1 mg fructose/min.) throughout the experiment, an additional 3.7 mg glucose/min. was provided shortly after the stimulation began and for the remaining time.

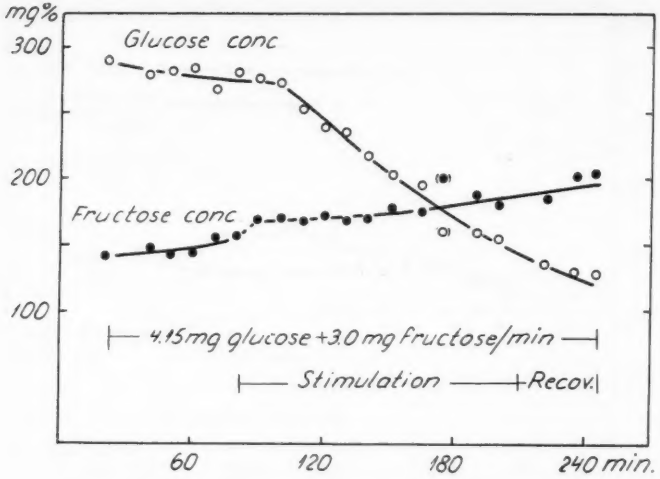

Fig. 6. *Effect of muscular exercise upon glucose and fructose uptakes.*
Weight of hind limb preparation 1,300 g. Perfusion begun and priming dose of fructose (550 mg) added at zero time. Continuous addition of glucose and fructose as indicated. Oxygen consumption: 2.9 ml/min. before, and 14 ml/min. during stimulation.

Evidently, this supplementary glucose was not quite adequate to counterbalance the increased rate of uptake during stimulation.

A better compensation was attained in the experiment of Fig. 8 in which the slope of the glucose concentration curve in the stimulatory period remained approximately the same as in the latter part of the equilibration period.

From these and other similar experiments it appears then that at serum glucose levels around 200 mg% muscular exercise of the intensity here employed causes a rise in the rate of glucose uptake of about 3 mg/min./kg hind limb weight or almost a doubling of the resting uptake.

In both the latter experiments a recovery period was included (in which the supplementary glucose infusion was still maintained). From the course of the glucose concentration during these intervals it appears that the increased rate of glucose uptake is maintained for some time after the cessation of stimulation.

Again, it appears in Figs. 7 and 8 that no increase in fructose uptake is brought about by muscular exercise.

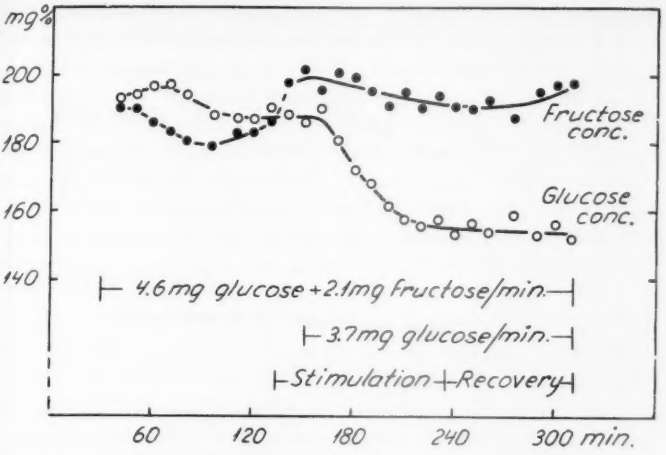

Fig. 7. *Effect of muscular exercise upon glucose and fructose uptakes.* Weight of hind limb preparation 1,400 g. Perfusion begun at zero time, priming dose of fructose (800 mg) added shortly after. Continuous additions of glucose and fructose as indicated. Oxygen consumption: 3.0 ml/min. before, and 10.5 ml/min. during stimulation.

Discussion.

The observation in the present study that insulin increases the rate at which galactose is lost from the extracellular space of perfused hind limb preparations of cats, together with the previous finding in this laboratory (GAMMELTOFT et al. 1944) that insulin is without effect upon the rate of utilization of fructose in the same preparation, confirms the results obtained by LEVINE and coworkers in eviscerated, nephrectomized dogs.

Similarly, our findings that muscular exercise increases the rate at which glucose and galactose are lost from the extracellular space, while it has no such effect on fructose, are confirmatory to GOLDSTEIN et al.'s (1953) results in the eviscerated dog.

The fact that the confirmations were obtained with the less complex hind limb preparation further supports LEVINE et al.'s assumption that muscle cells are the site of the selective effects on the uptake of various hexoses. Insulin and muscle activation apparently have the same pattern of effect and non-effect on the uptake of various hexoses.

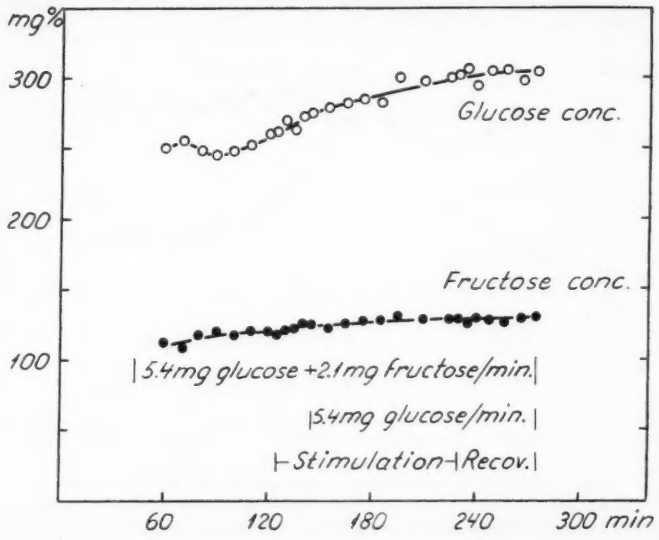

Fig. 8. *Effect of muscular exercise upon glucose and fructose uptakes.*
Weight of hind limb preparation 1,380 g. Perfusion begun at zero time, priming dose of fructose (700 mg) added 15 minutes later. Continuous additions of glucose and fructose as indicated. Oxygen consumption 3.3 ml/min. before, and 14 ml/min. during stimulation.

The problem of which processes are involved in the transfer of glucose into muscle cells has received increasing attention in recent years. Any hypothesis on the nature of this transfer must obviously take into account the existence of selective effects on the transfer of different hexoses. On the other hand, knowledge of such effects is not in itself sufficient to provide a solution of the problem.

The possible ways of transfer of hexoses into muscle cells may roughly be classified as (1) passage in chemically unmodified form through the cell boundaries, (2) uptake comprising a chemical conversion (especially formation of complexes) of the hexoses in the cell boundaries.

If the cell boundaries were completely impermeable to hexoses as such and the formation of chemical complexes essential to their transfer, the selective effects of insulin (and activation of muscles) could be easily explained by assuming it increased the rate of complex formation of some hexoses but not that of others.

The occurrence of a phosphorylation as a link in the transfer of glucose and its acceleration by insulin has been proposed as a possibility by LUNDSGAARD (1949). The assumption of such a mechanism has, however, as pointed out by LEVINE, the disadvantage that the ability of insulin to enhance the transfer of galactose cannot be accounted for by the same fundamental effect, since, apparently, muscle does not contain galactokinase. At present no other complexes than phosphate esters are known to be formed directly from hexoses in animal tissues. Hexose-dehydrogenase, known to be present in liver, has not been demonstrated in muscle.

If hexoses crossed the boundaries of muscle cells in the free state, free hexoses should be present in the interior, although of course, in the case of glucose, the concentrations might be held quite low, due to the presence of glucokinase.

LEVINE et al. (1950) have claimed that they were able to recover administered galactose completely from the carcasses of their dogs, the insulin-treated as well as the non-treated. Consequently, they feel that galactose was actually present as such upon its transfer to the interior of the cells. In a single stimulation experiment we tried to measure the reducing power of a Somogyi filtrate prepared from a glucose oxidase-treated "brei" of muscle (quadriceps) obtained at the end of the distribution period. Taken at their face value the results indicated that galactose was present in muscle in amounts corresponding to those computable from the final plasma concentration and the final distribution volume. The results must however be considered with much reserve, since the reducing power was only some 2.8 times higher than that (49 mg% "galactose") of muscle from cats to which no galactose had been administered. More specific methods, such as chromatography, are obviously needed to answer the question.

PARK (1953) has recently reported experiments in which intracellular concentrations of glucose were measured under various conditions; unfortunately, only an abstract and a somewhat more extensive reference (STADIE 1954) of this work is available. According to these sources intracellular glucose could be demonstrated in rat diaphragms exposed *in vitro* to sufficiently high external glucose concentrations; if insulin was also present intracellular glucose was demonstrable at lower external concentrations. When incubations were made at low temperatures intracellular glucose could be demonstrated at appreciable lower ex-

ternal glucose concentrations. Free intracellular glucose was also demonstrated in diaphragms removed from insulin-treated, eviscerated rats, but not in diaphragms obtained from animals not treated with insulin.

These findings are important, since they are quite compatible with the view that glucose is transferred as such across the cell boundaries and that insulin somehow decreases their resistance to this transfer. Confirmation of PARK's results is therefore desirable, and the more so because they are in disagreement with those of LUNDSGAARD (1939), who failed to demonstrate any increase in total reducing power of muscles of insulin-treated perfused hind limb preparations over that of muscles from preparations not treated with insulin.

The question may justly be raised whether the view of a transfer of hexoses in a chemically unchanged form is compatible with great differences in the rates of transfer of various hexoses and with the selective effects of insulin on such transfers. In the case of red cells related findings have recently been accepted as evidence for the participation of a membrane carrier system with associated coupling and uncoupling enzymes in the process of sugar uptake. (LEFEVRE 1954.) In our opinion it seems however too early to dismiss entirely the possibility of a transfer of hexoses as such. Such transfer might be imagined as occurring through minute "channels" offering different degrees of steric hindrance to the passage of various substances, depending not only on their molecular sizes, but also on their detailed, spatial structure. Also, such channels, or possibly better, lattices, would not necessarily have to be looked upon as static systems; they might be fluctuating and might even offer unequal resistances to the passage of substances in different directions. The laws describing the rates of passage of substances through such systems would obviously be fundamentally different from those applying to free diffusion. It appears moreover, that facilitating and inhibitory effects of various substances on transfers, as well as mutual inhibitions of hexose transfers should be explainable in such systems without resorting to enzymes as the sites of action.

The question of mutual inhibition in uptakes of hexoses by muscle cells has been touched upon by LEVINE et al. as well as WICK and DRURY. While the former express the opinion that the levels of blood glucose appear without relation to the effect of insulin on galactose uptake, the latter interprete their data as

indicating that high levels of glucose depress the uptake-promoting effect of insulin on galactose.

Since it appeared to us that inhibition of glucose uptake by galactose might be responsible for some of the symptoms of infants suffering from galactosaemia, a few experiments were made to decide whether such an inhibition could be demonstrated in muscle cells.

Two rat hemidiaphragms were incubated for three hours at 37° C in 2 ml of phosphate buffered salt solution (with an ionic composition as used by PEARSON et al. 1949) containing 1 unit of insulin, 500 mg% galactose and 150 mg% C^{14}-labelled glucose. Controls, without galactose in the medium, were run simultaneously. In none of three experiments, did we observe any significant difference in (1) disappearance of glucose from the medium, (2) production of $C^{14}O_2$ or (3) post-incubation glycogen contents.

These negative results do not, of course, exclude the possibility that galactose may inhibit the uptake of glucose by other tissues.

As mentioned above we have observed an increase in the rate of glucose uptake in the hind limb preparations during muscular exercise. With the intensity of stimulation used the rates of additional glucose uptakes were evaluated to be some 3 mg/min./kg at plasma glucose levels around 200 mg%. The simultaneous rises in oxygen uptakes amounted to some 7 ml of oxygen/min./kg. Since only some 2 ml of oxygen is required for complete oxidation of 3 mg of glucose, it is evident that the additional uptake of glucose during stimulation was much too low to cover the increase in oxygen consumption; other substances must have served as the main sources for the increase in metabolic rate. The R. Q. values observed during stimulation also deserve mention in this connection. Admittedly, such R. Q. values based upon single determinations of arterial-venous differences for CO_2 and O_2 must be interpreted with caution. Nevertheless, the fact that they were always found quite low (range 0.60—0.84, in six experiments) strongly suggest that muscles possess a considerable capacity for fat oxidation.

The rise in the rate of glucose uptake during stimulation is still quite considerable when compared with the uptake in the resting state, and it appears furthermore that it commences quite soon after the stimulation has begun; it is of course to be expected that it should take some time before a reduction in the glucose concentration in the immediate vicinity of the muscle

cells could lead to a drop in the glucose concentration of the blood plasma.

The occurrence of a considerable and rapidly established rise in glucose uptake by muscle upon their activation, must obviously be followed in the intact animal by an increased output of glucose from the liver, since only moderate reductions occur in the plasma glucose concentration during muscular exercise. At present we have no definite knowledge of the factors responsible for such compensatory reactions. Epinephrine, of course, has to be considered, but, as touched upon in a previous paper (KRUHØFFER and MUNTZ 1954) it does not seem unlikely that changes in the metabolic state of, say, muscle cells make them release substances which cause alterations in the metabolic machinery of the liver, resulting *inter alia* in an increased output of glucose. Variation in the production of such factors is also a potential explanation of the metabolic "defects" observed by CHAIKOFF, CHERNICK and co-workers (cf. *e. g.* CHAIKOFF 1952) in liver slices from starved and diabetic rats, of the changes in uptake/output of glucose by the liver occurring during a glucose-tolerance test (SOSKIN et al. 1938), of secondary effects (glycogen formation, decrease in ketogenesis) of insulin on the liver metabolism, etc.

Summary.

Insulin has been found to increase the rate at which D-galactose is lost from the extracellular space of perfused hind limb preparations of cats.

Electric induction of muscle contractions in the same preparation also raised the rate of disappearance of D-glucose and D-galactose from the extracellular space, but had no such effect on D-fructose.

These and previous findings in this laboratory confirm those obtained by LEVINE, GOLDSTEIN et al. on eviscerated, nephrectomized dogs and thus support the view that insulin and activation of muscle exert selective effects on the uptake of various hexoses by muscle cells.

The mode of uptake of sugars by muscle cells is discussed, and it appears that it is still impossible to decide whether sugars can cross the cell boundaries in the free state or only by way of some chemical transformation.

While not at all sufficient to cover the augmented metabolic

rate, the rise in glucose uptake during muscular activation was still quite appreciable (viz. of the same magnitude as the rate of uptake in the resting preparation) and in the intact organism it must be met by increased glucose output from the liver. The nature of such compensatory reactions is still unknown.

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From the Institute of Neurophysiology, University of Copenhagen and the Department of Ear-, Nose- and Throat-Diseases, University Hospital of Copenhagen.

The Influence of Acetylcholine, Menthol and Strychnine on Taste Receptors in Man.

By

ARNE P. SKOUBY and K. ZILSTORFF-PEDERSEN.

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It has previously been demonstrated that cold and pain receptors in the human skin (BING and SKOUBY 1950, SKOUBY 1951, 1953), thermal receptors of the cat's tongue (DODT, SKOUBY and ZOTTERMAN 1953), baroceptors of the carotid sinus of the cat (LANDGREN, SKOUBY and ZOTTERMAN 1953) and chemoceptors of the olfactory zone in man (SKOUBY and ZILSTORFF-PEDERSEN 1954) are sensitized to their adequate stimuli by small amounts of acetylcholine, while larger amounts exert an inhibiting effect. Acetylcholine in itself does not evoke sensations or electrical activity in afferent nerve fibres.

Recently LANDGREN, LILJESTRAND and ZOTTERMAN (1954) have demonstrated that acetylcholine-like substances influence the impulse traffic evoked in the lingual branch of the glossopharyngeal nerve when tap water or hypertonic NaCl-solutions are ejected over the surface of the tongue. The substances in themselves did not evoke impulses in agreement with the findings quoted above. The effect is most likely due to an action on specific taste fibre endings in spite of the fact that differentiated endings have never been demonstrated in the frog tongue. In the present work the influence of acetylcholine on the sensitivity of the highly differentiated taste receptors in man was investigated.

Menthol influences thermoceptors (HENZEL and ZOTTERMAN 1951, DODT, SKOUBY and ZOTTERMAN 1953), baroceptors and chemoceptors of the carotid area in the cat (LANDGREN, SKOUBY

and ZOTTERMAN 1953) and smell receptors in man (SKOUBY and ZILSTORFF-PEDERSEN 1954) in a similar way as acetylcholine and the effect on taste receptors was investigated as well.

Finally, the effect of strychnine on taste receptors was examined, as the sensitizing effect of this substance on the chemoceptors of smell (SKOUBY and ZILSTORFF-PEDERSEN 1954) might be due to an action on their ganglionic cell properties and not on their receptor properties.

Technique.

The subjects were two female and two male students 24—28 years of age. None was a heavy smoker and they did not smoke before the experiments. Their sensitivity to taste stimuli was measured by the strength of an anodal current necessary to produce an acid taste sensation. Steel electrodes were used. The anode, with a contact area of 12 mm^2, was placed in the midline of the tongue near the tip, while the cathode was in contact with the buccal mucosa. The taste threshold was determined as the mean value of the lowest current eliciting and the highest not eliciting acid taste sensation when the anode was in contact with the tongue for approximately two seconds. The current was varied in steps of 4 μA and 0.5 minutes allowed between the determinations. The resistance between the electrodes during the application was approximately 1,000 Ω and variations in the resistance of the tissue produced only inconsiderable variations in the strength of the current due to a series resistance of 24,000 Ω in the circuit. As seen from the diagram (fig. 1) the strength of the current is calculated from the potential difference in Volt read on the instrument V and a resistance of approximately 25,000 Ω.

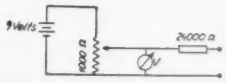

Fig. 1. Arrangement for evoking taste sensation by anodal current.

The spontaneous taste threshold variations were investigated for periods of up to one hour on different days. When uniform values were obtained over a 15 minute period, spontaneous changes did not exceed 10 per cent during the subsequent hour. The threshold values varied from day to day and from individual to individual (table 1).

The solution to be tested was dropped on the tip of the tongue by a pipette (0.005 ml; 37°C).

Water free acetylcholine was dissolved immediately before use in 0.9 per cent NaCl solution to give a concentration of 0.1 μg to 1 mg per ml. Stock solutions of menthol (0.04 per cent) in water and strychnine nitrate (1 per cent) in 0.9 per cent saline were diluted with 0.9 per cent saline to give the desired concentrations.

For controls 0.9 per cent NaCl was used.

Table 1.

Taste threshold at the tip of the tongue in μA in two subjects on eight different days.

Experimental days		1	2	3	4	5	6	7	8
Subjects	Me.	108	60	90	86	80	86	76	90
	Mu.	142	180	86	122	86	102	118	86

Results.

Acetylcholine in amounts of 1—100 μg per ml decreased the taste threshold by 20—63 per cent. In most experiments the threshold decrease was preceded by a transient increase of up to 30 per cent. There was no correlation between the concentrations applied and the change in threshold (fig. 2). Smaller (0.1 μg per ml) and large amounts (1 mg per ml) produced a transient increase of the taste threshold of up to 40 per cent but never decreases exceeding 10 per cent of the initial value. The threshold change lasted 5 to 35 minutes depending on the amounts applied and the individual tested.

Saline solution (0.9 per cent) produced increases of up to 114 per cent but never decreases (fig. 2).

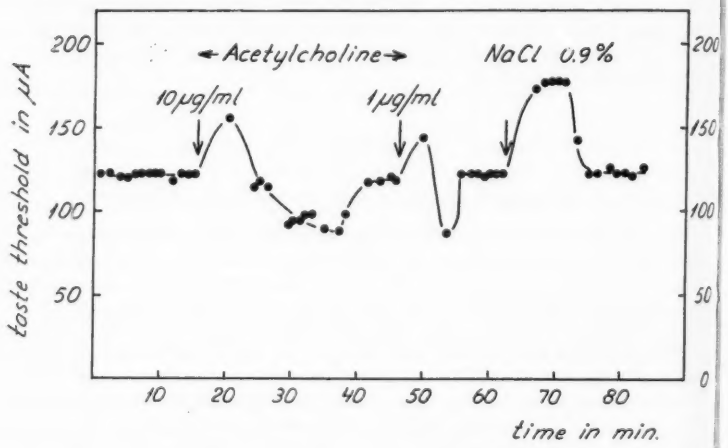

Fig. 2. Taste threshold variations in subject Mu. before and after the application of acetylcholine (10 μg/ml and 1 μg/ml) and 0.9 per cent saline to the tip of the tongue at the times indicated by arrows.

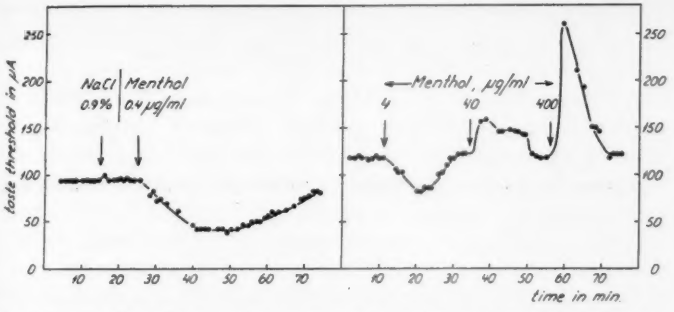

Fig. 3. Taste threshold variations in subject R. before and after the application of saline and menthol (0.4—400 μg/ml) to the tip of the tongue at the times marked by the arrows.

Menthol in amounts of 0.4—4 μg per ml decreased the taste threshold by up to 60 per cent. Larger amounts increased the threshold by up to 60 per cent (fig. 3). With 40—400 μg a decrease in threshold was found in one individual only and always preceded by an increase.

Strychnine nitrate was applied in amounts of 0.1 to 10 mg per ml. In most experiments transient increases of up to 40 per cent were produced. No decreases exceeding 10 per cent of the initial value occurred (fig. 4). Thus, the effect of strychnine did not deviate from that of the solvent.

Acetylcholine produced no taste sensations in itself. Menthol in strong concentrations (40—400 μg per ml) produced the cool taste of menthol for the first minutes after application, while all concentrations of strychnine used produced a bitter taste persisting up to half an hour when the strongest solutions were used.

Discussion.

The present experiments demonstrate that acetylcholine in suitable amounts produces a significant decrease of the taste threshold. The effect may be due to an action on the receptor cells directly or indirectly. In experiments on the thermoceptors of the human skin and of the cat's tongue vascular effects were absent or not related to the sensitizing action (Bing and Skouby 1950, Dodt, Skouby and Zotterman 1953). Furthermore, neither acetylcholine nor menthol influenced the discharge from thermal

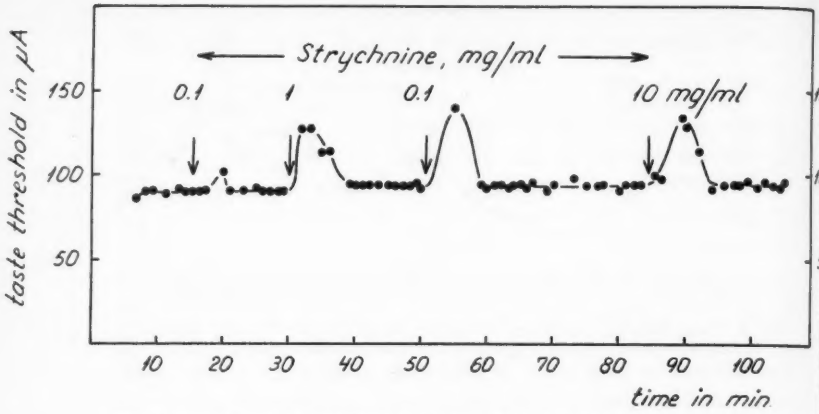

Fig. 4. Taste threshold variations in subject Me. before and after the application of strychnine (0.1—10 mg/ml) to the tip of the tongue at the times indicated by the arrows.

receptors when applied to the afferent nerve fibres of the cat's tongue as close as possible to their endings (DODT, SKOUBY and ZOTTERMAN 1953). Therefore, assuming similar conditions in the present experiments, the effect of acetylcholine is most likely a direct one on the receptors. A similar action may be assumed for menthol, which previously has been found to influence thermo-, baro- and smell receptors and the chemoceptors of the carotid body as does acetylcholine.

The strychnine experiments indicate that this substance does not influence receptor structures and that the threshold decreasing effect on smell receptors (SKOUBY and ZILSTORFF-PEDERSEN 1954) is probably due to their ganglion cell properties.

In high concentrations all the substances tested produced increases in threshold. Since the solvent often raised the threshold in a similar way, this effect cannot be attributed to an inhibitory effect of stronger concentrations of the test substance as was found in previous investigations on thermo-, baro- and chemoceptors. Thus, the present finding adds the taste receptor to the list of the receptors on which acetylcholine has a sensitizing but not stimulating effect independent of the structure or adequate stimulus of the peripheral receptor (SKOUBY 1951, 1952).

The present results agree with electrophysiological studies on taste receptors of the frog tongue (LANDGREN, LILJESTRAND and

ZOTTERMAN 1954). Their findings with decamethonium or succinylcholine applied to the lingual mucosa agree with our finding on pain receptors (SKOUBY 1953) that these substances in contrast to curarine are synergistic to acetylcholine in its effect on peripheral receptors. Similarly GRANIT, SKOGLUND and THESLEFF (1953) found that the amount of tension of a muscle preparation determined whether the effect of succinylcholine on the impulse traffic in spindle fibres was absent or present, again indicating that acetylcholine-like substances are sensitizing but not in themselves stimulating. When a stimulating effect on the receptors (SCHWEITZER and WRIGHT 1938) or on peripheral neural junctions in the carotid body (LILJESTRAND et al.) is claimed, the adequate stimulus has to be abolished during the action of the substance. However, in all electrophysiological studies published, the neurograms demonstrate activity before the test period (EULER, LILJESTRAND and ZOTTERMAN 1941, LILJESTRAND 1952, LANDGREN, LILJESTRAND and ZOTTERMAN 1952, 1954, LILJESTRAND and ZOTTERMAN 1954).

Summary.

Acetylcholine (1—100 μg/ml) decreased the taste threshold by 20—63 per cent as did *menthol* (0.4—400 μg/ml). Higher concentrations of these substances as of strychnine in concentrations of 0.1—10 mg/ml produced increases of the same order as those produced by the solvent.

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From the Cardiological Laboratory, Department of Medicine, Allmänna Sjukhuset, Malmö, and the University of Lund, Sweden.

Comparative Studies on Temperature Effects upon the Electrocardiogram in some Vertebrates.

By

GUNNAR BIÖRCK and BENGT JOHANSSON.

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In recent years, induced hypothermia has attracted much interest as a technical method facilitating surgical interventions in an "empty" and quiet heart. Several groups have already been able to transfer their work from the laboratory to the operating theatre with considerable success. Still, however, many — perhaps most — basic problems remain unsolved. Since 1952, we have been interested in the differences between natural hibernation and artificial cooling. In the course of our early experiments on hedgehogs, which are the only hibernators in our country that lend themselves to studies of the kind we have attempted, we found it of interest to compare the temperature effects on the electrocardiogram with corresponding data from non-hibernating mammals, like the dog and the pig, as well as from poikilothermic animals like the turtle, the frog and the fish. We have also had occasion to study electrocardiograms from humans, who were brought to our hospital with subnormal rectal temperatures. Some of our data will be presented below.

Material and Methods.

Experiments were performed on the following animals: *6* mongrel dogs, *9* hedgehogs (Erinaceus europaeus), *12* south african frogs (Xenopus laevis) and *8* flounder-fishes (Pleuronectes flesus L). Electrocardiograms on dogs and hedgehogs were recorded with a sensitive

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amplifier electrocardiograph (Elema Triplex) and on frogs and fishes with a four times more sensitive machine (Elema Junior). Needle electrodes were used in all instances. In dogs, leads corresponding to human leads were registered, with the dogs lying on their left side. In hedgehogs, who were generally rolled up, electrodes were placed in left and right scapular and left gluteal regions. In frogs only lead I was registered, with the electrodes stuck into the proximal part of their anterior extremities. In fishes, one electrode was placed 0.5 cm dorsally and slightly caudally of the insertion of the pectoral fin of the pigmented side, the other one 0.5 cm ventrally and slightly caudally of that point on the same side. The first electrode, then, was in the region of the arterial bulb, the latter in that of the venous sinus. The electrodes were arranged so that a higher potential in the first region would give an upward deflection of the ECG.

The fishes and the hedgehogs generally did not move much and their electrocardiograms did not show much disturbances. Some frogs, however, had to be slightly anesthetized with ether. The dogs were anesthetized with intravenous pentothal (11.5—40 mg/kg body weight) and, after intubation, with ether, nitrous oxide and oxygen in a closed or half-open system.

The temperatures were measured in centigrades by means of a thermocouple. In frogs and fishes, temperature changes were induced by placing the animals in water-baths of different temperatures. The temperature of frogs was measured with an electrode deposited 1—2 cm upwards in the cloaca. This was not applicable in our fishes; here, the electrocardiograms were recorded, when the animal had been staying in the water-bath for 10—15 minutes. Hedgehogs were taken from their out-door cages to a room of 19°—22° temperature, and their temperatures recorded with an electrode deposited dorsally in the skin. The dogs were cooled — and re-warmed — in a specially designed refrigerator box (BIÖRCK et al. 1954). Their temperatures were recorded by means of an electrode, deposited at least 10 cm upwards into the rectum.

The induction of temperature changes was arrived at in the following way:

Dogs were cooled in the aforementioned box and rewarmed in the same box. One dog was cooled twice. Electrocardiograms were recorded from 39°—34° and followed down to temperatures between 25°—19°.

Hedgehogs were all warmed from the hibernating state. 6 animals were warmed twice. The initial temperature varied between 4° and 13.°5. Warming was finished at temperatures varying between 27° and 33°. Temperatures were measured with somewhat irregular intervals on an average 8 times during the warming period. The observations are brought together in groups with mean temperature values of 7.°5, 12.°5, 17.°5, 22.°5, 27.°5 and 32.°5.

Frogs were all placed in water and warmed from a base temperature of 5°. 9 animals could be followed up to 35°, while 3 died between 30° and 35°. Electrocardiograms were recorded at 5-centigrade-intervals.

Also the fishes were placed in cold water and warmed from 5°. In one fish, warming started at 10° and in another one at 15°. Two fishes died between 20°—25°, five between 25°—30° and one between 30°—35°.

Results.

Fig. 1 shows typical average electrocardiographic complexes from the various species at different temperatures. The amplitude of ECGs from frogs and fishes are increased fourfold in comparison to those of dogs and hedgehogs. The complexes within frames are those obtained at the animals' "normal" temperature, when in full activity. From dog and hedgehog only lead II is represented. The leads from frog and fish are described in the text.

The complexes drawn in fig. 1 are based on mean durations of P, P—R, QRS, Q—T and T and mean amplitudes of P, QRS and T. Only in frogs, at high temperature, was an exception made for the T wave. This was in some cases positive, in others negative, giving an isoelectrical mean, which, however, did not occur in any tracing. Here, we have depicted only the mean of the largest group, which was the positive one.

The heart rate.

As shown in fig. 2 C, in dogs there was a decrease in heart rate from 146 beats/minute at 39° to 32 beats/minute at 19°. Depending on the degree of shivering, there was some initial increase in heart rate at the induction of hypothermia. This factor prob-

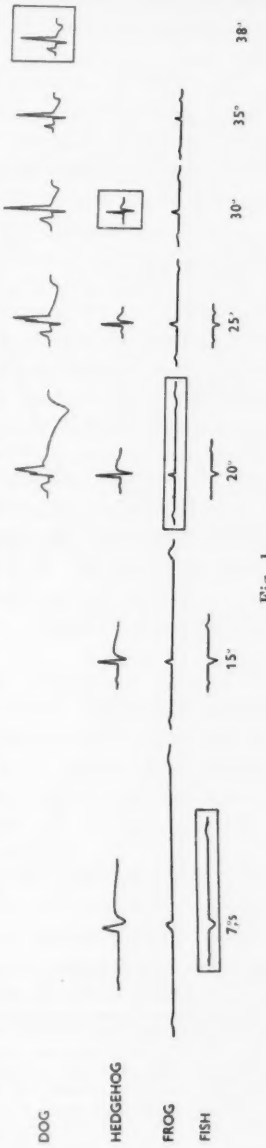

Fig. 1.

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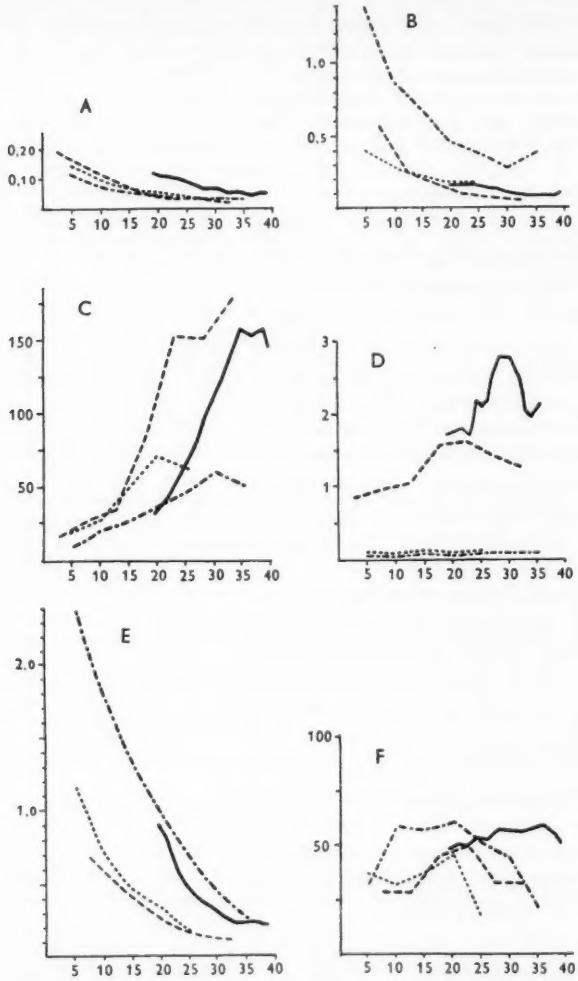

Fig. 2. A. QRS duration at different temperatures. Ordinate: QRS in seconds. B. P—R interval at different temperatures. Ordinate: P—R in seconds. C. Heart rate at different temperatures. Ordinate: heart rate in beats/min. D. QRS-amplitude at different temperatures. For hedgehogs the figure shows the mean amplitude of lead 2. For dogs only lead 2 of one dog is depicted. See further the text. Ordinate: QRS-amplitude in mV. E. Q—T interval at different temperatures. Ordinate: Q—T in seconds. F. Q—T in % of R—R at different temperatures. Ordinate: Q—T in % of R—R. Abscissa: temperature in °C in all figures.

———— Dog — · — · — · — Frog
— — — — — Hedgehog ..... Fish

ably explains some of the variations in values reported by other authors (HOOK et al. 1941, PREC et al. 1949, HEGNAUER et al. 1949, LANNEK 1949, HEGNAUER et al. 1950, BIGELOW et al. 1950, HORWITZ et al. 1953, CHURCHILL-DAVIDSON et al. 1953). In our material, like in those of some other authors (BIGELOW et al. 1950, OSBORN 1953), it was found that this initial increase was almost or completely abolished, when the dogs were in deep anesthesia. At lower temperatures the values given by most authors are more consistent and in good agreement to our findings.

In hedgehogs, we observed that the increase in heart rate during awakening showed a steeper slope in the region 15°—22.°5. A similar observation was made on marmots by ENDRES et al. (1931), whereas SARAJAS (1954) failed to observe any such phenomenon in his four hedgehogs (of which only two were in natural hibernation), nor did CHATFIELD and LYMAN (1950) find it in golden hamsters. The findings of SARAJAS (1954), that the correlation between heart rate and temperature became less evident at lower temperatures than at higher ones was confirmed in our series. The absolute values at 7.°5 — 26 beats/min. — and at 32.°5 — 181 beats/min. — are in good agreement with those of other authors (SARAJAS 1954, SUOMALAINEN et al. 1951). At the lowest temperature, 3°, we recorded a heart rate of 15 beats/min., but we also registered heart rates of 5—15 beats/min. at temperatures between 9° and 17°.

The frogs showed an increase from 8 beats/min. at 5° to 61 beats/min. at 30°. In only two frogs did the heart rate increase up to a temperature of 35°. In the others the heart rate again decreased, in one case between 20° and 25°, in three cases between 25° and 30° and in 6 cases between 30° and 35°. — The rate of increase was irregular, and such maxima at 15° and 25° as described by TAYLOR (1931) were not observed.

In fishes, there was an increase in heart rate from 19 beats/min. at 5° to 71 beats/min. at 20°. In one case there was a turning-point already at 15°, in three others at 20° and in still two it came first at 25°. At all temperatures, our values are higher than those given by BRITTON (1923—24) for different kinds of fish (elasmobranchii and teleostomi). The difference was greater with increasing temperature.

Cardiac rhythm.

Regular sinus rhythm was prevailing in the dog. In the two dogs, who were cooled to about 20°, there were frequent hetero-

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tope ventricular premature beats as well as QRS complexes of varying shape, with P—R intervals of different duration. In this temperature range, we did not encounter ventricular fibrillation.

In hedgehogs, sinus rhythm with an easily discernible P wave was prevailing between 15° and 25°. Below and above this range, P was often difficult to identify, and sinus arrhythmia was common.

In fishes and frogs, P was sometimes difficult to identify. Ventricular premature beats were frequent at 35° in frogs, otherwise there were no indications in these species that the sinus rhythm was suppressed.

The P wave.

In the dog, the P wave all through the temperature range studied was positive and slightly notched. The amplitude, which was at 38° 0.40 mV — a value three times less than that given by LANNEK (1949) for non-anesthetized dogs — rose to 0.55 mV at 30° and, then, again decreased, being at 20° 0.30 mV.

The hedgehog's P wave was sometimes very flat and difficult to ascertain, particularly at the lowest and highest temperatures (7° and 30°). There was often sinus arrhythmia. In the rapid phase, P was often positive, whereas it was isoelectric or negative in the slow phase. The amplitude was low in all instances, as an average 0.05 mV. For this reason, the variations in amplitudes were not measured, as the errors must be quite big. This does also apply to frogs and fishes. The P wave in frogs was positive, with an average amplitude of 0.02 mV. In fishes, it was negative with the leads applied by us, and had an amplitude averaging 0.01 mV.

With increasing temperature and heart rate, the duration of the P wave became shorter. In dogs, P had a mean duration of 0.11 sec. at 20° as against 0.05 sec. at 38° — the latter value falling between those given by LANNEK (1949) and HORWITZ (1953). In hedgehogs, the duration was 0.10 sec. at 7.°5 as against 0.03 sec. at 30°; in frogs the corresponding values at 5° and 30° were 0.20 sec. and 0.07 sec. with a small increase at 35°, to 0.09 sec. In fishes, the duration was 0.08 sec. at 5° and 0.04 sec. at 25°.

In five dogs of six, the P wave showed a deformity which appeared between 32° and 28° and again disappeared below 24°. This deformity (figs. 1 and 3) consisted in an initial notching of the P wave, with an amplitude of 0.1 mV and a duration of 0.02 sec. This deformity was most easily observed in lead II, and was seen also at rewarming in two of the animals.

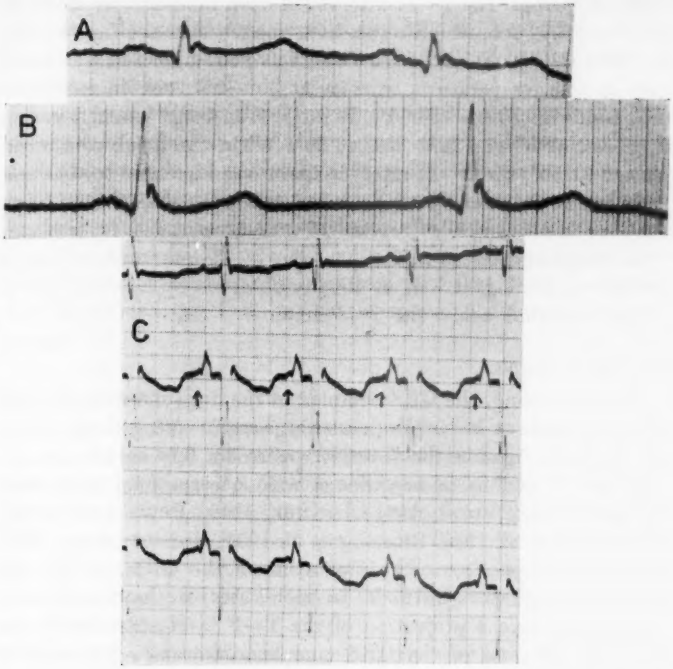

Fig. 3. A and B show ECGs from two adult human beings with a rectal temperature of about 30°. Note the "Osborn wave", the long Q—T interval and the slow heart rate. — The figures show the second standard lead only.
C shows the three standard leads of an ECG from a dog at a rectal temperature of 28°. The arrows show the initial notching of the P wave. Also note the well marked "Osborn wave" in leads II and III.
The time distance between two heavy lines is 0.1 sec.

P—R interval.

As shown in fig. 2 B, the P—R interval always was prolonged with lower temperatures and slower heart rate. In dogs, the value at 38°, 0.09 sec., is in conformity with those given by most other authors (HOOK et al. 1941, LANNEK 1949, HEGNAUER et al. 1950, BIGELOW et al. 1950), whereas HORWITZ (1953) gave a value of 0.12 sec. At lower temperatures — about 22° — P—R was somewhat shorter — 0.16 sec. — than values given by other authors (HOOK et al. 1941, HEGNAUER et al. 1950, BIGELOW et al. 1950).

In hedgehogs, the P—R interval in deep hibernation, at about 7.°5, was 0.57 sec. which was a little longer than the figure given by SARAJAS (1954), although there was good agreement in heart rate at this temperature, whereas at 30°—35°, our value of 0.06 sec. conforms to his. The P—R interval in frogs varied between 1.38 sec. at 5° and 0.28 sec. at 30°. When the heart rate again slowed at 35°, the P—R interval again was increased to 0.38 sec.

In fishes, P—R at all temperatures was lower than that of frogs: 0.40 sec. at 5° and 0.17 sec. at 25°.

In hedgehogs, frogs and fishes, the P—R interval was always isoelectric. In dogs it was, as already observed by LANNEK (1949), often somewhat below the T—P line.

The QRS complex.

During cooling, the QRS complex in the dogs showed increased notching and, at lower temperatures, became successively wider. As shown in fig. 2 A the *duration* was at 38° 0.06 sec. and at 20° 0.12 sec., which is in accordance with observations from other authors (HOOK et al. 1941, LANNEK 1949, PREC et al. 1949, HEGNAUER et al. 1950, BIGELOW et al. 1950, HORWITZ et al. 1953). At lower temperatures, the separation of the QRS and ST segments became more difficult. In such cases, we have considered the point where a projection of the T—P level cuts the R or S wave as the end of the QRS complex. However, the so-called "Osborn wave" (see below) has always been considered as belonging to the S—T segment.

In the hedgehogs, the same problem of separation occurred, and was solved in the same way. The duration of QRS was here at 7.°5 0.15 sec. and at 32.°5 0.03 sec., which is in accordance with those values given earlier (SARAJAS 1954).

In frogs the corresponding values were: at 5° 0.12 sec. and at 35° 0.04 sec. In fishes, the values were: 0.15 sec. at 5° and 0.04 sec. at 25°.

Hedgehogs, frogs and fishes did not show any increased notching at lower temperatures.

The *amplitude* of QRS in lead II (fig. 2 D) was not constant in the dogs. However, a maximum was generally encountered between 28° and 32°.

In hedgehogs, in 7 of 9 animals, or altogether 11 times of 15, the QRS_{II} amplitude had a maximum of 1.65 mV at 22.°5 (observation in late February). A minimum was observed at 7.°5,

with an amplitude of 0.85 mV, and at 32.°5 with 1.25 mV. When the same determinations were made late in November, at the beginning of the hibernating period, only 3 out of 7 animals showed a similar phenomenon.

In frogs and fishes, the QRS amplitude did not show any consistent tendency, but was always very much inferior to that of dogs and hedgehogs, generally around 0.1 mV.

The *form* of the QRS complex in dogs varied considerably, particularly with regard to the existence of Q and S waves. Hedgehogs had either a QR or a RS complex. In frogs, likewise, complete QRS complexes rarely occurred. In fishes, most complexes may be termed QS, *i. e.* one single, negative deflection was recorded.

ST—T pattern.

In dogs, the ST—T pattern became disturbed at about 33°. The typical change was a small, positive wave immediately after the end of the QRS complex. It was seen earliest in lead II. Initially, its duration was 0.02 sec., but as the temperature decreased, the wave gained in duration and amplitude. This particular wave is already described by OSBORN (1953), who maintains, that it appears at 25°. As stated, it was seen already at higher temperatures in our dogs.[1]

Apart from the "Osborn wave" there were several other distorsions of the ST—T pattern. Already after some few degrees' cooling it became impossible to separate the ST segment and the T wave. They became fused into a slowly descending limb, which later on abruptly rose to the isoelectric level. This picture is similar to that described by BIGELOW et al. (1950) in their dogs. Occasionally, this pattern was reversed, with a slow ascent to the isoelectric level. Sometimes there was a tray-shaped deformation. These changes were most obvious in leads II and III and often combined with an increased negativity of T, particularly below 22°. When the cooling was not that low, similar patterns were, however, regularly observed during rewarming in the region between 24°—30°.

[1] A similar wave was seen in the electrocardiogram from two adult patients, who were admitted to the department of Medicine, Allmänna Sjukhuset, Malmö with a rectal temperature of about 30° (fig. 3). Their electrocardiograms also showed bradycardia with sinus or nodal rhythm, ventricular premature beats and a prolonged Q—T time. In a boy, aged 2 1/2 years, who was operated on in hypothermia because of a cyanotic congenital heart disease, and was cooled down to 27°, no "Osborn wave" was seen.

In the hedgehogs, the ST segment was, generally, not measurable. In those instances, where a measurement was possible, which was most often at low temperature, they never exceeded 0.08 sec. The T waves did not show any negativity at lower temperatures. The changes in the T waves consisted of an increase of duration, from 0.09 sec. at 30° to 0.53 sec. at 7.°5, and a small variation in amplitude similar to that of the QRS_{II} amplitude: 0.18 mV at 30°, 0.20 mV at 20° and 0.16 mV at 7.°5. Frequently, there was in the lower temperature ranges a minimal depression of the level immediately following T, lasting almost to the next P wave.

In frogs, the duration of T was prolonged at low temperatures, being 0.07 sec. at 35° and 0.25 sec. at 5°. The appearance of T varied much, with positive, diphasic and negative T waves. Positive waves were more frequent at lower temperatures, whereas at 25° 5 animals had a negative T wave and 4 a diphasic one. At 30°—35° T was frequently not seen, but when it was visible it was most often positive. In the temperature range of 30°—35° there were also deviations of the ST segment upwards in 2, and downwards in 1 animal. Otherwise, it remained at the isoelectric level.

Also in fishes, T was wider at lower temperature: 0.07 sec. at 25° and 0.15 sec. at 5°. T, that was always positive at low temperatures (*i. e.* discordant, as QRS was negative with the leads used), became negative at 25° in two animals, while it was positive in one and could not be recognized in the others. The ST segment was, generally, isoelectrical.

Q—T interval.

The variations in Q—T interval (fig. 2 E) showed the widest range of all the portions of the electrocardiogram. This was true for all animal species.

The values in dogs — 0.21 sec. at 38° and 0.85 sec. at 20° — are in agreement with those given by other workers (LANNEK 1949, HORWITZ et al. 1953, HEGNAUER et al. 1950, BIGELOW et al. 1950).

In hedgehogs, the Q—T intervals at the extreme temperatures — 0.11 sec. at 32.°5 and 0.68 sec. at 7.°5 — were in agreement with those given by SARAJAS (1954), while in the middle temperature range, our values exceeded his.

In frogs, the Q—T interval was 2.36 sec. at 5° and 0.67 sec. at

25°. These values exceed the corresponding values for fishes which were 1.16 sec. at 5° and 0.18 sec. at 25°.

Discussion.

The changes in the electrocardiogram, that occur following great alterations of the body temperature, may be due to several factors. Different rates of cellular metabolism, variations in the activity of other enzymatic processes and of humoral transmittor substances as well as changes in electrolyte composition etc. may affect the electrical processes in the myocardium, of which the electrocardiogram gives an integrated picture. We deliberately abstain at this moment from the venture of a unifying interpretation of our findings.

However, the animals we have used in our studies, represent different classes among the vertebrates, having also a different way of responding to changes in environmental temperatures. Generally, increasing body temperature brings about an elevation of the heart rate. Obviously, this is not true above certain temperatures, beyond which life is increasingly disagreeable to poikilothermic animals like frogs and fishes. It was of interest to observe in our fishes, that the decrease in heart rate appeared at temperatures, where there was still an increase in respiratory movements. On some other fishes, however, BRITTON (1923—24) has observed a simultaneous reduction in respiratory and cardiac activity at about 28°.

As for the various components of the electrocardiograms, we have already mentioned a peculiar change at the beginning of the P wave in dogs, different from the normal notching, as shown in figs. 1 and 3. At cooling of a pig, there was no initial elevation, but a rather sharp initial negative deflection of the P, of about the same amplitude and duration as in the dog. This change appeared at 25°. None of the patients with hypothermia previously mentioned did show any P wave deformity, but their body temperatures were not lower than 30°.

The amplitudes of the different waves in the electrocardiogram varied somewhat irregularly in the frogs and fishes. The variations in dogs and hedgehogs, however, were quite regular. The amplitude of QRS in lead II in dogs had its maximum between 28° and 32°. This was also true of the P wave, whereas no such

differences were found for the T wave. BIGELOW (1950) found a maximal amplitude at about 24°, below which temperature it in most cases decreased. We have found no other mention of this change. RICHARDS et al. (1953) found that the amplitude of all waves in the ECG increased when mice were exposed to cold, CRISMON (1944) has published corresponding results with rats.

In hedgehogs, likewise, there was an increase in QRS and T wave amplitudes at awakening from hibernation in the spring, with a maximum about 20°. The change was more marked for QRS than for T. At awaking from hibernation in November, only 3 hedgehogs out of 7 showed this variation. SARAJAS (1954) found no noteworthy changes in amplitude in his hedgehogs at warming, which took place after two weeks' natural hibernation, and in August, when he made them hibernate with insulin.

The shape of the T wave varied much. In fishes, it was very low and difficult to distinguish, and generally discordant at low, but concordant at higher temperatures. The opposite was more or less true for the frogs. The hedgehogs never showed any inversion of the T wave, whereas in dogs T became progressively negative as the body temperature was lowered, particularly so in the region of 20° (fig. 1).

OSBORN (1953) has already described a change in the beginning of the ST segment in hypothermia, consisting of a positive, rounded wave immediately following QRS. This phenomenon, which we have called the "Osborn wave", was found in six out of seven of our dogs, although already at 33° as against 25° in OSBORN's series. OSBORN attaches a serious prognostic importance to this finding. However, four dogs of six in our series had an uncomplicated recovery. The "Osborn wave" appears at a time, when ventricular excitability is increased, according to ORIAS et al. (1950), as indicated by HEGNAUER (1954).

The duration of the various components of the electrocardiogram, individually and in relation to each other, varied much in the animal species studied. The heart rate was lower and P, P—R and Q—T intervals had a longer duration in frogs than in fishes at the same temperature, while the QRS was shorter. Q—T in % of R—R (fig. 2 F) was higher in frogs than in fishes. When the heart rate again decreased at higher temperatures, Q—T and consequently, Q—T in % of R—R, decreased in both frogs and fishes, while P and PR increased in duration in frogs but not so in fishes.

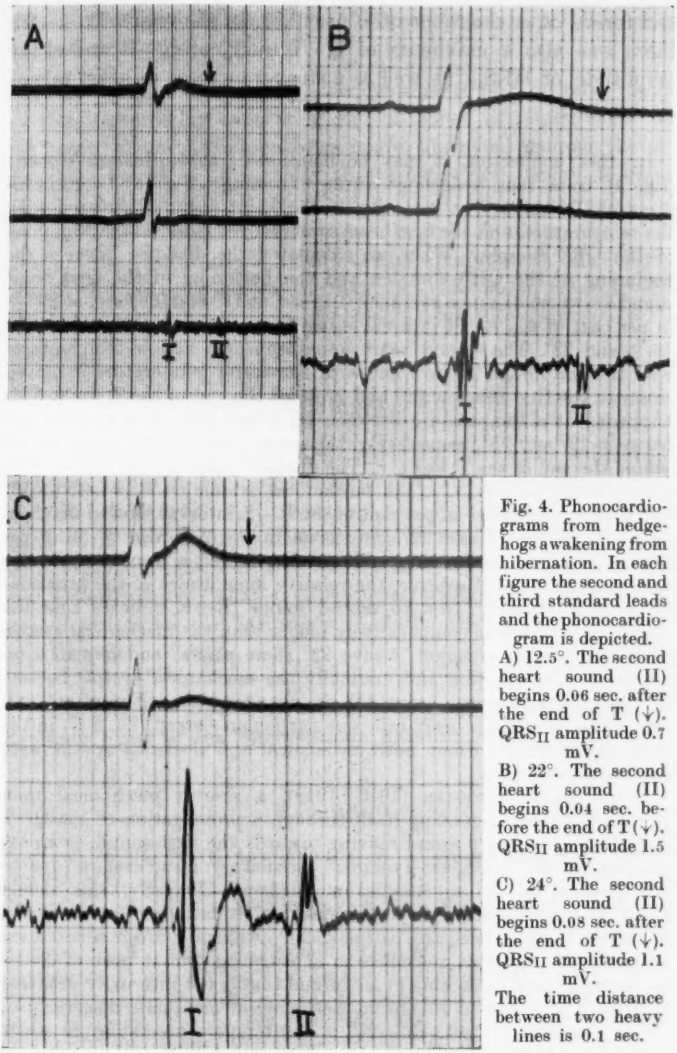

Fig. 4. Phonocardiograms from hedgehogs awakening from hibernation. In each figure the second and third standard leads and the phonocardiogram is depicted.
A) 12.5°. The second heart sound (II) begins 0.06 sec. after the end of T (↓). QRS_{II} amplitude 0.7 mV.
B) 22°. The second heart sound (II) begins 0.04 sec. before the end of T (↓). QRS_{II} amplitude 1.5 mV.
C) 24°. The second heart sound (II) begins 0.08 sec. after the end of T (↓). QRS_{II} amplitude 1.1 mV.
The time distance between two heavy lines is 0.1 sec.

In hedgehogs, the duration of P—R, QRS and Q—T increased considerably at lower temperatures. The rate of increase was most marked in QRS and QT in the temperature range of 12.°5—20°

and for P—R in the range of 7.°5—12.°5. In the region 12.°5—20° there was also a maximum of Q—T in % of R—R and of the amplitude of QRS_{II}, as well as a steeper increase in heart rate at warming.

In this connection it may be mentioned, that *phonocardiograms* (fig. 4) from four hedgehogs during warming all showed certain interesting features: The intensity of the heart sounds increased with the temperature, and the first heart sound moved closer to the beginning of the QRS complex. When we calculated the distance between the beginning of the QRS complex and the beginning of the first heart sound — which distance averaged 0.15 sec. at 14° and 0.03 sec. at 29° — in per cent of the R—R interval — we found maximal values between 16.°5 and 22°. The second heart sound appeared at low temperatures *after* the end of the T wave but with increasing temperature it moved closer to the first heart sound and *preceded* the end of the T wave. This happened at a temperature between 16.°5 and 22°. At a temperature between 22° and 25°, respectively, the second heart sound again moved *behind* the end of the T wave. — The distance between the first and the second heart sounds, *i. e.* the mechanical systole — 0.28 sec. at 16° and 0.15 sec. at 29° — in per cent of the R—R interval showed maximal values between 15° and 22°. As shown in fig. 2 F also Q—T in per cent of R—R, *i. e.* the electrical systole, showed a maximum in this temperature range. However, the relative magnitudes of the variations of Q—T/R—R and "first to second sound"/R—R indicate that the electrical systole was comparatively longer than the mechanical systole in this temperature region. Above 22° there was a tendency to a reversal of this condition and gradually the mechanical systole became the longer one, whereas below 15° the changes observed were not uniform. The increase in heart rate was particularly steep (fig. 2 C) in the temperature range, where both mechanical and electrical systole were relatively prolonged.

According to HEGGLIN (1946, 1949), a shorter mechanical than electric systole, manifesting itself through a prolongation of the Q—T and the second heart sound moving towards the first sound, is an expression of an "energetic-dynamic" circulatory insufficiency, due to disturbances of the myocardial metabolism. Clinically, this should occur in hypocalcemia, diabetic coma and some other intoxications. While such conditions are pathological, awakening from hibernation normally is a healthy procedure in a hedgehog, but it may of course be, that induced awakening in an animal that is not internally prepared to it, will give rise to somewhat pathological metabolic disturbances. — As a matter of fact, 15°—20° is the temperature range, in which ventricular fibrillation is most apt to occur during cooling of other homiothermic animals (BIGELOW et al. 1950, LYNN et al. 1954, JUVENELLE et al. 1952). GRAUER and HEGGLIN (1946) and RICHARDS et al. (1953) have, furthermore, shown that cooling in rabbits and mice, respectively, does not cause any movement of the second heart sound

towards the first heart sound. The same was true in experiments that we have performed on pigs.

These studies, of necessity, are incomplete and do only touch on some interesting aspects of the influence of temperature on the heart. From the comparisons made, however, emerges the remarkable power of adjustment that hibernators display as compared to other animals, homiothermic or poikilothermic. The secrets of hibernation in this respect may be found in the myocardium itself, in humoral agents acting upon it or in an integration of the nervous control mechanism — or at all these levels. Further attempts must be directed at all these targets.

Summary.

Electrocardiograms have been recorded for dogs, hedgehogs, frogs and fishes at different temperatures. The variations in heart rate, P—R interval, QRS complex, Q—T interval, and Q—T in % of R—R are presented in graphs. Some peculiar electrocardiographic alterations, like the initial P wave disturbance and the "Osborn wave", that appear during cooling are discussed in some detail. Some comparisons with electrocardiograms in human hypothermia are made.

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Department of Physiological Chemistry, University of Lund, Lund, Sweden.

Intestinal Absorption of Taurocholic Acid in the Rat.

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JAN SJÖVALL and INGRID ÅKESSON.

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Using various methods based on colour reactions many authors have been able to show that there is a rise in portal cholate concentration during the absorption of cholate from the small intestine (GREENE, ALDRICH, ROWNTREE 1928, JOSEPHSON and RYDIN 1936, JENKE and GRAFF 1939). JOSEPHSON and RYDIN did not find any bile acids in the lymph and concluded that the bile acids are transported via the portal veins. New possibilities of studying the problem of bile acid absorption have come up with the synthesis of carboxy-labelled bile acids (BERGSTRÖM, ROTTENBERG and VOLTZ 1953). This paper reports some results on the intestinal absorption of labelled taurocholic acid in the rat.

Experimental.

Rats weighing 200—300 g were used. The main intestinal lymph duct was cannulated as described by BOLLMAN et al. (1948). Bile fistulas were done according to BERGSTRÖM, SJÖVALL and VOLTZ (1953). The animals were kept in restraining cages similar to those used by BOLLMAN et al. (1948). The lymph fistula animals had free access to food but as they sometimes developed diarrhea and were apt to drink excessively, they were given 0.64 per cent sodium chloride in restricted amounts. Animals with both lymph and bile fistulas almost never got

diarrhea and they had free access to food and 0.9 per cent sodium chloride.

Labelled biosynthetic taurocholic acid was prepared by intraperitoneal injection of 2 mg cholic acid-24-^{14}C to a rat with bile fistula. The bile was collected and immediately frozen to — 16°C. The labelled material in this bile was taurocholic acid 98—99 per cent and glycocholic acid 1—2 per cent (NORMAN 1954). 2—3 ml of this bile together with $^1/_2$—$^1/_4$ ml corn oil was given per os to the bile fistula animals at least 48 hours after the operation. The activity excreted in the bile fistula was followed by direct plating of bile on copper planchets placed on a hot plate. The copper planchets were changed each ten minutes. To follow the activity appearing in the lymph it was necessary to use the same method although an infinitely thin layer on the copper planchets could not be obtained. To get the lymph as evenly distributed as possible on the planchets the lymph drops were allowed to fall in a drop of water that was placed on the hot copper planchet. Extraction of the bile acids from the lymph was done with alcohol : ether 3 : 1.

Results.

1. Rats having lymph and bile fistulas.

Two rats having both a bile and an intestinal lymph fistula were given bile with labelled bile acids (between 20,000 and 30,000 c.p.m.) per os. The activity very rapidly appeared in the bile and only traces of activity was found in the lymph (Table 1). The same rapid excretion in the bile was found in a rat that had only a bile fistula. 67 per cent was excreted during the first hour and 29 per cent during the second hour after the administration per os of 3 ml/^{14}C/bile + 0.5 ml corn oil.

Fig. 1 shows the recovery of radioactivity in bile and lymph

Table 1.

Recovery of bile acids in bile and intestinal lymph after administration per os of bile containing labelled bile acids 20,000 to 30,000 c.p.m. (mainly as taurocholic acid).

Rat	Days after operation	Given per os	Per cent of administered dose recovered in		
			Bile 0—1 hr.	1—2 hr.	Lymph 0—2 hr.
GLR 1	8	3 ml/^{14}C/bile + 0.5 ml corn oil	70	17	1
GLR 2	2	2 ml/^{14}C/bile+0.25 ml corn oil	80	5	0.1

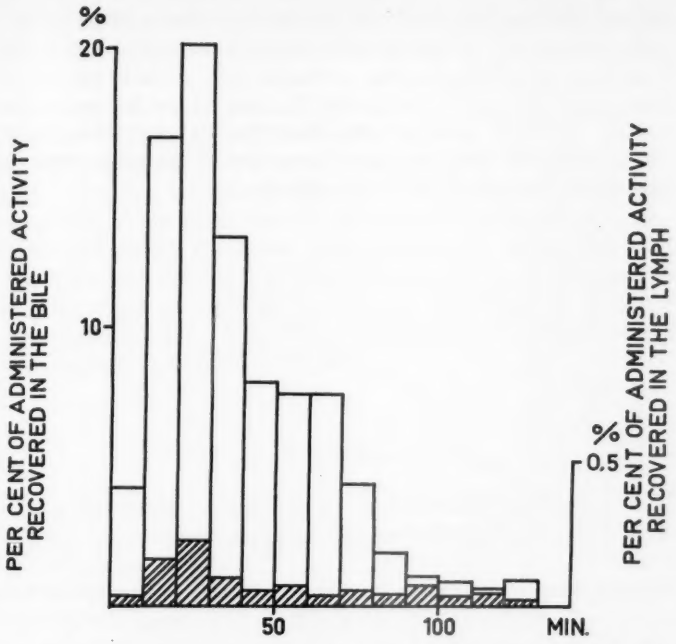

Fig. 1. Activity recovered in the bile and the lymph of a rat given bile with labelled bile acids per os. Bile □, lymph ▨.

in rat GLR 1. As was the case with rat GLR 2 the maximum excretion occurred between 20 and 30 minutes after the administration of bile and this was true for the excretion through both fistulas.

Faeces from rat GLR 2 (Table 1) was collected during the three days following the administration of bile and after refluxing three times for 2 hours with boiling ethanol the activity was measured on a suitable aliquot of the extract. The activity that had not come out in the bile and lymph was recovered in the faeces.

2. Rats having only the intestinal lymph duct cannulated.

A rat having an intestinal lymph fistula was given 0.4 ml bile containing labelled biosynthetic taurocholic acid (664,000 c.p.m.) and 0.25 ml corn oil per os 72 hours after the operation. The activity in the lymph was measured (fig. 2) by direct plating of

ten minute fractions 0—2.5 hours and 6—8 hours after the administration. The lymph portions between 2.5 and 6 hours and between 8 and 24 hours were extracted with alcohol : ether 3 : 1 and a suitable aliquot was plated (0.2 mg fat per sq. cm of the copper planchet; BERGSTRÖM, BLOMSTRAND and BORGSTRÖM 1954). It is seen that very small amounts of bile acids were excreted continuously in the lymph fistula.

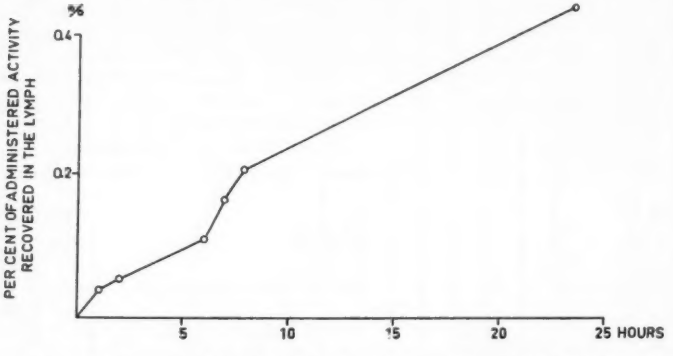

Fig. 2. Activity recovered in the lymph of a rat given bile with labelled bile acids per os.

Discussion.

After administering the bile from a rat that had been given cholesterol-4-^{14}C to a rat with lymph fistula SIPERSTEIN et al. (1952) could recover only 2—8 % of the given activity in the lymph. When labelled cholesterol is given to bile fistula rats the major part of the activity excreted in the bile is found as bile acids (see for example BERGSTRÖM 1954), a minor part is, however, found in the unsaponifiable fraction and this part probably accounts for the activity found in the lymph by SIPERSTEIN et al. Our results show that only minute amounts of the taurocholic acid appear in the intestinal lymph. That the activity recovered in the lymph was taurocholic acid is made very probable by the fact that taurocholic acid remains practically unchanged during the entero-hepatic circulation (SJÖVALL, to be published). Practically no splitting of the conjugation takes place in the small intestine by the action of bacteria or pancreatic enzymes.

The absorption takes place very rapidly as has already been

shown by GREENE et al. (1928) in bile fistula dogs. No conclusions as to the time of entero-hepatic circulation of the bile acid molecules can, however, be drawn by these experiments, because of the abnormal state which is induced by the continuous withdrawal of all the bile acids formed by the liver.

The maximum of the activity appearing in the lymph coincides with the maximum excretion in bile. This would indicate a leakage of bile acids between the blood and the lymph as possibly occurs with the serum proteins (BORGSTRÖM and LAURELL 1953). In rats with lymph fistulas there is also a continuous loss of very small amounts of bile acids in the lymph as would be expected when the bile acids are continuously absorbed from the intestine and reexcreted in the bile.

Summary.

The intestinal absorption of biosynthetic taurocholic acid labelled in the cholic acid part has been studied in animals with bile and lymph fistulas.

The absorption was very rapid and most of the administered activity was excreted in the bile during the first hour. Practically no taurocholic is transported via the lymph vessels since only traces of activity were found in the intestinal lymph.

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SJÖVALL, J., to be published.

Department of Physiological Chemistry, University of Lund, Lund, Sweden.

Intestinal Absorption of Cholanic Acid in the Rat.

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The long chain fatty acids are transported almost quantitatively as triglycerides via the lymphatic pathway after they have been absorbed from the small intestine. Taurocholic acid after absorption is carried to the liver with the portal vessels (JOSEPHSSON and RYDIN 1936) and only traces of activity are found in the intestinal lymph when labelled taurocholic acid is administered per os (SJÖVALL and ÅKESSON 1955). It was therefore of interest to study the absorption of the unsubstituted cholanic acid, an acid with solubility properties similar to those of the long chain fatty acids but with the bile acid ring structure.

Experimental.

The cholanic acid-24-^{14}C used was material synthesized by BERGSTRÖM, ROTTENBERG and VOLTZ (1953).

Male rats weighing 200—300 g were used. The main intestinal lymph duct was cannulated as described by BOLLMAN et al. (1948). Bile fistulas were done according to BERGSTRÖM, SJÖVALL and VOLTZ (1953). The animals were kept in restraining cages of a type similar to that described by BOLLMAN et al. (1948). Animals having only a lymph fistula were given a solution of 0.64 per cent sodium chloride;

animals having both a bile and a lymph fistula were given 0.9 per cent sodium chloride. All animals had free access to food.

24—48 hours after the operation, the rats were given the labelled cholanic acid dissolved in 0.25 ml corn oil by gastric intubation under a light ether anesthesia. Animals having a bile fistula were given 2 ml of their own bile in addition. The bile was collected in ethanol and the activity was determined by direct plating of a suitable aliquot. The lymph was collected in a flask at room temperature.

The lymph samples were immersed in a twentyfold amount of ethanol : ether 3 : 1, boiled, filtered, evaporated in vacuo and the lipids extracted with several portions of chloroform. The chloroform extract was dried over anhydrous sodium sulphate, evaporated in vacuo and weighed. The lipids were dissolved in a suitable volume of chloroform and the activity determined by plating an aliquot giving about 0.2 mg fat per sq. cm of a copper planchet. In some instances, the activity was followed at ten minutes intervals by direct plating of bile and lymph. Infinite thinness of lymph on the copper planchets could not be obtained but the lymph was allowed to fall in a water droplet that was placed on the planchet in order to get the lymph as evenly distributed as possible. Separation of lymph lipids was done according to BORGSTRÖM (1952 a, b).

Results.

Absorption of cholanic acid in the intact rat.

The determination of the amount of cholanic acid absorbed in the intact rat after administration of cholanic acid-24-^{14}C per os presents some difficulties. At the same time as unabsorbed activity appears in faeces the absorbed part is taken up by the liver and excreted in the bile whereby some of this part is lost via the faeces. It has been shown, however, that in animals with bile fistulas the main part of the activity appearing in faeces after peroral administration of cholanic acid-24-^{14}C is unchanged cholanic acid. (BERGSTRÖM and SJÖVALL 1955.) At the same time it was shown that cholanic acid after intraperitoneal administration is excreted in the bile mainly as compounds more polar than cholanic acid. These compounds are conjugated with taurine in the bile but in the intestinal tract the conjugation is split by the action of intestinal bacteria. These facts make it possible to determine the amount cholanic acid which has not been absorbed by the determination of unchanged cholanic acid in faeces after separation of the acids with reversed phase chromatography (BERGSTRÖM and SJÖVALL 1951, SJÖVALL 1953). A simpler method, however, giving an approximate estimation

Table 1.

Estimation of intestinal absorption of cholanic acid in the intact rat.

"Cholanic acid" = Petrol ether phases; "Hydroxy acids" = ethanol phases (see text). Cholanic acid absorbed = 100—percentage of "cholanic acid" in faeces.

Rat no.	Per cent of administered activity excreted in faeces as "cholanic acid" (Ch) and "hydroxy acids" (Hy) during day								Approximate amount of cholanic acid absorbed. Per cent
	1		2		3		4—5		
	Ch	Hy	Ch	Hy	Ch	Hy	Ch	Hy	
1	16	1	19	14	4	13			61
2	16	7	6	11	3	9	3	15	72
3	15	15	7	14	1	13	1	7	76

of the amount of unchanged cholanic acid in the faeces is to perform a two stage counter-current distribution of an acidified faeces extract between petrol ether and 70 per cent ethanol. The cholanic acid will stay in the petrol ether phase and the more polar acids in the ethanol phase although some mixing of the acids will occur. This method was used for the determination of the intestinal absorption of cholanic acid in the intact rat (Table 1). The rats were given about 1 mg of cholanic acid-24-^{14}C in 0.25 ml corn oil by gastric intubation. Faeces was collected each day and refluxed twice for 2 hours with boiling ethanol. In the counter-current procedure 100 ml of each phase was used and the activity of the phases was determined by plating an amount giving 0.2 mg sq. cm of a copper planchet. Approximately 70 per cent of the administered amount was absorbed.

Rats with the intestinal lymph duct cannulated.

Four rats having the main intestinal lymph duct cannulated were given 0.5—1 mg cholanic acid-24-^{14}C dissolved in corn oil. Table 2 shows that the percentage of the administered dose found in the intestinal lymph is very low. Although conclusions are drawn from these figures it must be realized that the fat absorption in lymph fistula rats may differ somewhat from that in intact rats. The lymph lipids were separated after extraction of the lymph. The percentage for the fat fractions of the total amount of radioactivity found in the lymph was found to be: Phospholipids 15—18 per cent, glycerides 26—61 per cent and free acids 22—56 per cent. As the lymph, however, had not been

Table 2.

Recovery of cholanic acid-24-^{14}C in the intestinal lymph after administration per os.

Rat no.	Sacrificed after	Per cent of administered dose found in the lymph	Per cent of the administered dose found in faeces
LR 6	18 hours	6	20
LR 7	22 »	7	29
LR 8	3 days	3	40
LR 9	3 »	3	[1] 73

[1] This rat developed a slight diarrhea.

frozen immediately after collection the free acids found might have been formed through autolysis and too much importance must not be attached to these figures.

Rats having both the main intestinal lymph duct and the bile duct cannulated.

As the recovery of cholanic acid in the intestinal lymph was very low, two animals with both a lymph and a bile fistula were given labelled cholanic acid to estimate the proportion between the amount of activity recovered in the lymph and the amount recovered in the bile (Table 3). The animals were given 2 ml of their own bile together with the fat mixture. The amount of cholanic acid absorbed was low compared with the values for the normal rats. Fig. 1 shows that the excretion of activity in the bile is very slow. This must mean that the absorption takes place very slowly since it has been found that when the cholanic acid is administered intraperitoneally it is excreted very rapidly

Table 3.

Recovery of activity in rats having both a bile duct fistula and a lymph duct fistula after administration of cholanic acid per os.

Rat	Sacrificed after	Per cent of administered activity found in		Per cent of administered activity found in faeces	Total amount of administered activity recovered
		Lymph	Bile		
GLR 3	3 days	[1] 2	[1] 23	70	95
GLR 4	3 »	3	25	73	101

[1] Per cent excreted during the first 30 hours.

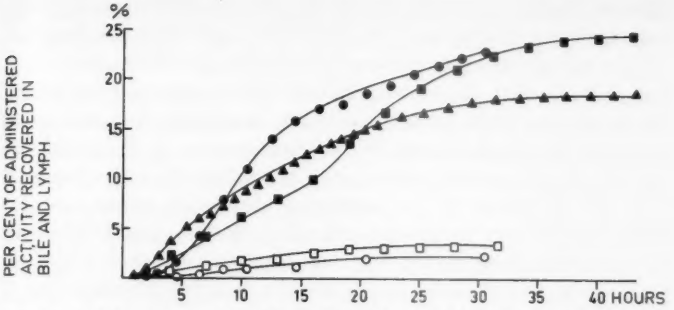

Fig. 1. Activity recovered in bile and lymph after administration of labelled cholanic acid.

GLR 3: bile (●) lymph (○) (see Table 3).
GLR 4: bile (■) lymph (□).
Rat having only a bile fistula: bile (▲).

in the bile as more polar compounds conjugated with taurine (BERGSTRÖM and SJÖVALL 1955, NORMAN 1954). In fig. 1 there is also shown the excretion of activity in the bile of a rat having only a bile fistula and given the same amount cholanic acid as the animals having both a lymph and a bile fistula. It must be emphasized that in animals with bile fistulas the fat absorption is abnormal (see discussion).

Discussion.

After absorption from the small intestine the long chain fatty acids are transported via the lymphatic pathway. With decreasing chain length more of the acids are transported in the portal blood; of an absorbed amount of decanoic acid only 5—19 per cent is found in the lymph (BLOOM, CHAIKOFF and REINHARDT 1951) mainly in the glyceride fraction (BLOMSTRAND 1955). This indicates that the way of transport after absorption partly is determined by the solubility properties of the acid, more hydrophilic acids thus being transported mainly in the portal blood. Our results, however, indicate that the solubility properties of the acid are not the only factor governing the route of transport after the absorption, since cholanic acid is a very lipophilic acid similar to long chain fatty acids in solubility properties and yet this compound is not transported appreciably via the lymph.

BORGSTRÖM (1954) has studied the in vitro incorporation of

different fatty acids into glycerides during hydrolysis by ratpancreatic juice. He found that cholanic acid was incorporated only to a very small extent as was also the case with cholic acid and butyric acid. The possibility of this being another factor governing the route of transport was discussed. Decanoic acid, however, was incorporated to the same extent as oleic acid. As the fat soluble cholanic acid was practically not incorporated into the glycerides it was concluded that there might exist an enzyme specificity which does not allow the incorporation of this bile acid structure into glycerides. This would agree well with the finding that cholanic acid is absorbed via the intestinal lymph only to a minor extent.

BERGSTRÖM, BORGSTRÖM, TRYDING and WESTÖÖ (1954) have shown that 2,2-dimethylstearic acid is absorbed mainly via the lymph as triglyceride. A minor part is found in the lymph as free acid (BLOMSTRAND and TRYDING 1955) and the acid can also be found to an appreciable extent in the portal blood (BLOMSTRAND and TRYDING 1955 b). BERGSTRÖM et al. found that the acid was not appreciably incorporated into glycerides by the action of ratpancreatic juice in vitro. It has also been shown (BLOMSTRAND and TRYDING 1955 a) that the acid remains free in the intestinal content in contradistinction to the unsubstituted long chain fatty acids. Still, after absorption, 2,2-dimethylstearic acid is found mainly in the glyceride fraction in the lymph. These results indicate that the glycerides have been formed in the intestinal wall by enzymes which are not hindered by the two methyl groups. Such a glyceride formation probably does not occur to any greater extent with the cholanic acid as it is mainly absorbed via the portal vessels where probably the lower fatty acids occur mainly as free acids as has been shown for decanoic acid (BORGSTRÖM 1955). Determination of the activity in different fat fractions in the lymph after administration of labelled cholanic acid showed that a small incorporation of cholanic acid into glycerides did take place but as the amount of cholanic acid transported in the lymph is very small the total incorporation into glycerides will be extremely small.

It would seem as if the route of transport of fatty acids after absorption in normal animals is partly determined by the solubility properties of the acid, partly by enzyme specificity. Decanoic acid is transported in vena porta in spite of the easiness with which it can be incorporated into the glycerides, and there

are enzymes in the intestinal wall that can incorporate the sterically hindered and more hydrophobic dimethyl stearic acid into the glycerides and allow transport of this acid in the lymph. That the structure of the acid is also of importance is shown in the case of cholanic acid which is transported mostly in the portal vessels. It is possible that the enzymes can not incorporate this acid into glycerides and that steric factors preclude the formation of cholanic acid glycerides.

In the absence of bile the mechanism of fat absorption is changed (SIPERSTEIN, CHAIKOFF and REINHARDT 1952, BORGSTRÖM 1953). Our results have shown that less cholanic acid was absorbed in the bile fistula animals than in normal animals. In our experiments the fat was given together with two ml bile but it is not clear whether the refeeding of this small amount of bile had any marked effect on the fat absorption in bile fistula rats. The bile acids were presumably very rapidly absorbed and excreted in the bile.

Summary.

The intestinal absorption of cholanic acid-24-^{14}C in rats has been studied. In the normal rat about 70 per cent of an administered amount is absorbed. In the rats with lymph fistulas 3—7 per cent of the activity was found in the lymph.

The amount of cholanic acid absorbed in rats having both a bile duct and a lymph duct fistula was less than in normal rats. About 10 per cent of the absorbed activity was found in the lymph, 90 per cent was excreted in the bile.

Factors governing the route of transport after intestinal absorption of fatty acids have been discussed.

The technical assistance of Mrs. Ann-Mari Andersson is gratefully acknowledged.

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Department of Surgery and Department of Physiological Chemistry,
University of Lund, Lund, Sweden.

Metabolism of Desoxycholic Acid in the Rabbit.

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By

PER-HUGO EKDAHL and JAN SJÖVALL.

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It has been shown that desoxycholic acid is converted to cholic acid in the rat (BERGSTRÖM, ROTTENBERG and SJÖVALL 1953). The rat bile does not contain detectable amounts of desoxycholic acid (< 2 per cent of total bile acids; BERGSTRÖM and SJÖVALL 1954) and it would probably be hydroxylated to cholic acid very rapidly if it were present. Therefore it is of interest to study the metabolism of desoxycholic acid in an animal where cholic and desoxycholic acids are present at the same time. In the rabbit the two acids occur together (desoxycholic acid predominating) conjugated mainly with glycine. (OKAMURA and OKAMURA 1930, SEKITOO 1931, KISHI 1936.)

Experimental.

White albino rabbits weighing 2—2.5 kg were used. The gall bladder was removed and a polythene cannula was inserted into the common bile duct. It was found very difficult to avoid a peritoneal irritation with minor infectious foci during the operation and the rabbits proved to be very sensitive and some animals died shortly after the operation.

After receiving the labelled acid intraperitoneally or intravenously as sodium salt in saline the rabbits were placed in cages of a type similar to that used for rats (BERGSTRÖM, SJÖVALL and VOLTZ 1953)

so that they could get greatest possible freedom without being able to remove the cannula. The animals had free access to food. The bile was collected in ethanol and small aliquots were plated on copper planchets (less than 0.2 mg per sq cm) for the determination of excreted activity.

Desoxycholic acid-24-^{14}C was the material synthesized by BERGSTRÖM, ROTTENBERG and VOLTZ (1953). Extraction of the bile acids excreted through the bile fistula was done with butanol according to NORMAN (1954). Reversed phase partition chromatography of conjugated bile acids has been described by NORMAN (1953). In some cases the conjugated bile acids were hydrolyzed in 1 N sodium hydroxide at 110° for 5 hours and after acidification and ether extraction the free acids were separated with reversed phase partition chromatography (BERGSTRÖM and SJÖVALL 1951, SJÖVALL 1953). Determination of the activity of the fractions from the chromatographies was done as described by BERGSTRÖM, SJÖVALL and VOLTZ (1953).

Paper chromatography of bile acids was carried out according to SJÖVALL (1952, 1954) and HASLEWOOD and SJÖVALL (1954).

Results.

A. Chromatographic analysis of rabbit bile.

The bile from a fistula was collected in alcohol during a period of approximately 20 hours. The alcohol was evaporated in an open dish on a steam bath and the bile solids were dissolved in water, acidified and extracted with butanol. Fig. 1 shows the titration curve of this extract run with reversed phase partition chromatography using phase system C (NORMAN 1953). The fractions containing the front band (180—400 ml effluent) were combined and evaporated. 1 mg was run on an ascending paper chromatogram using amylacetate : heptane 85 : 15 as moving phase and 70 % acetic acid as stationary phase (both phases equilibrated before use) (SJÖVALL 1955). After spraying with phosphomolybdic acid three spots were visible, one very weak running slightly slower than taurocholic acid, one running as taurodesoxycholic acid, and the third and strongest running somewhat slower than taurodesoxycholic acid and not quite separated from this acid. The spots may not necessarily be bile acids, since phospholipids for example can give the colour with phosphomolybdic acid (see also section for metabolism of desoxycholic acid).

The second band corresponding to glycocholic acid (B, 560—780 ml effluent) on paper chromatography only showed one spot at the same place as glycocholic acid.

The fractions of peak C (1,700—2,600 ml effluent) were combined and evaporated and the residue was extracted with butanol after acidification in the usual way. To remove the bromothymol blue the acid was run on a new column using phase system C and its position localized using the phosphomolybdic acid spot test. The fractions containing the acid were combined, the sol-

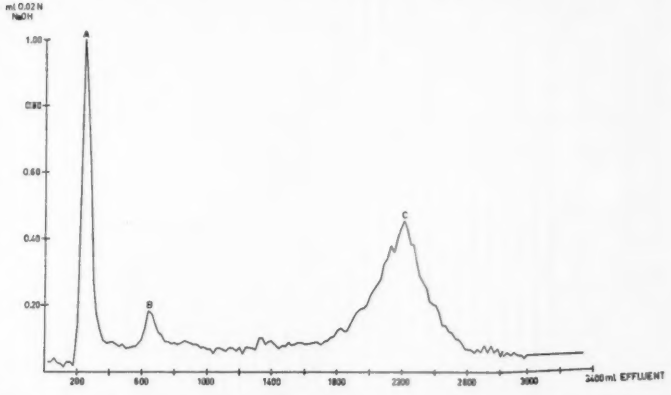

Fig. 1. Chromatography of the butanol extract of unhydrolyzed bile excreted during a period of 20 hours. Column 72 g hydrophobic Supercel. Phases. Type C (see text).

vents evaporated and the residue dissolved in ethanol. After addition of water to turbidity glycodesoxycholic acid crystallized in needles. (Melting point 183—184°, no depression with authentic glycodesoxycholic acid.) Descending paper chromatography of the crystals using isopropyl ether : heptane 60 : 40 as moving phase only gave one spot at the place for glycodesoxycholic acid. Paper chromatography of the mother liquor in the same way revealed two unidentified spots running very close to and not quite separated from glycodesoxycholic acid. No glycochenodesoxycholic acid was found. On direct paper chromatography of rabbit bile no free bile acids could be detected.

B. Fate of intraperitoneally administered desoxycholic acid 24-^{14}C.

A rabbit with a bile fistula was given 2 mg desoxycholic acid-24-^{14}C 24 hours after the operation. Using a fraction collector the bile was taken at 10 minute intervals. Each fraction was

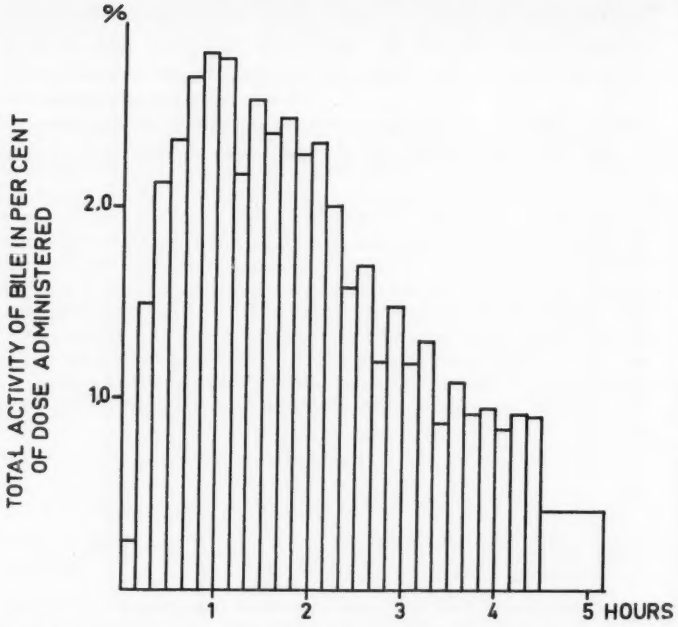

Fig. 2. Isotope excreted through bile fistula following intraperitoneal administration of 2 mg labelled desoxycholic acid.

made up to 10 cc with 80 % ethanol and 0.2 ml were plated on copper planchets for determination of activity. Fig. 2 shows the excretion of activity in the bile. This excretion is very slow compared with the curves obtained for rats given bile acids intraperitoneally. Probably the desoxycholic acid has been badly absorbed from the peritoneal cavity possibly as the cause of peritoneal irritation after operation. The rabbit died 6 hours after the injection of the bile acid. At autopsy only a small peritoneal irritation just below the liver could be found. The liver was ground in a Waring Blendor and refluxed on a water bath for two hours with three portions of ethanol. The activity of the extracts was determined. 48 % of the given amount had been excreted in the bile and 3.5 % was found in the liver.

The acids in the bile were extracted with butanol (NORMAN 1954) and subjected to reversed phase partition chromatography in phase system C (NORMAN 1953). The result is shown in fig. 3.

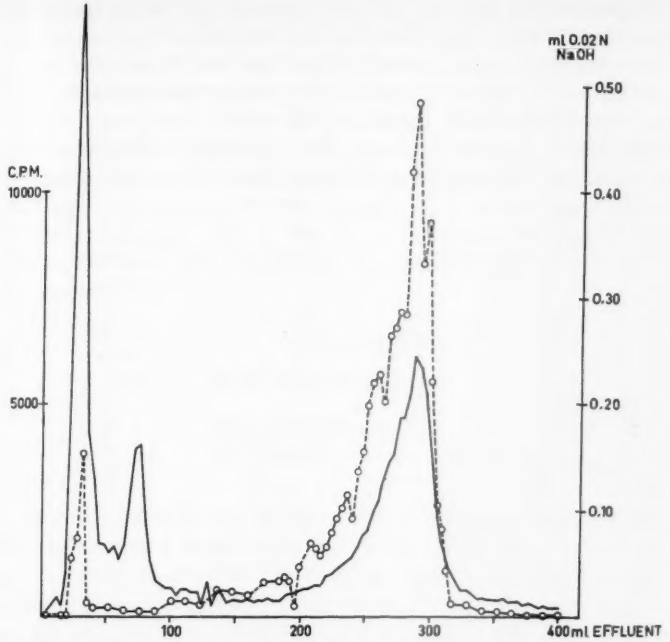

Fig. 3. Chromatography of butanol extract of unhydrolyzed bile excreted during 6 hours following intraperitoneal administration of 2 mg desoxycholic acid-24-^{14}C. Phases: Type C.

Most of the activity is present in the "glycodesoxycholic acid band". The fractions from this band were combined. Paper chromatography (ascending with isopropyl ether : heptane 75 : 25 as moving phase) showed only one spot of activity at the place for glycodesoxycholic acid. After butanol extraction of the residue 75 mg glycodesoxycholic acid was added as inactive carrier. Six recrystallizations from different solvents did not change the specific activity of the acid.

Approximately 4 per cent of the total activity came with the front and a paper chromatogram with the taurine system (SJÖVALL 1954) was run. The activity appeared at the same place as taurodesoxycholic acid. Reversed phase partition chromatography with butanol/water, however, revealed some inhomogenity of the material and further work has to be done to clear up the identity of this peak of activity.

About 10 per cent of the activity was found to be less polar than desoxycholic acid. This has not been found in any of the other experiments and possibly there has been a bacterial action on the acid in the abdominal cavity before the resorption (see for example SCHMIDT, HUGHES et al. 1942).

Finally it can be observed that no activity appears in the well-defined "glycocholic acid band" (50—100 ml effluent).

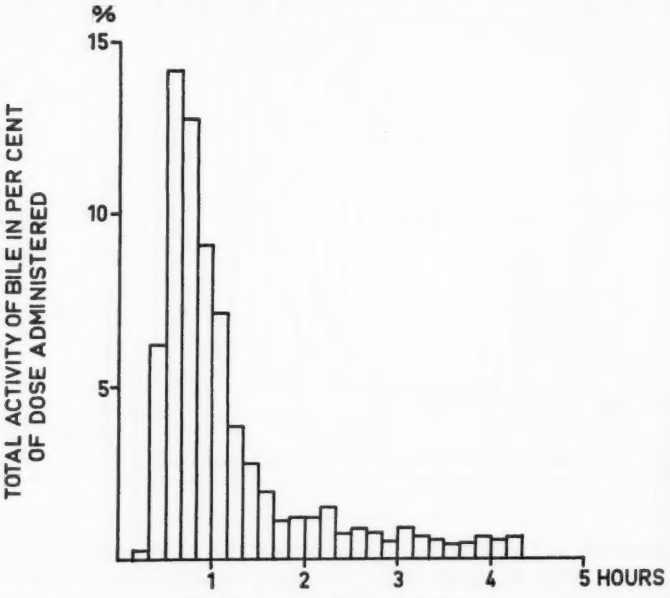

Fig. 4. Isotope excreted through bile fistula following intravenous administration of 1.45 mg desoxycholic acid-24-^{14}C.

C. Desoxycholic acid-24-^{14}C intravenously.

To avoid a possible contact with bacteria into the abdominal cavity labelled desoxycholic acid was administered to two rabbits by intravenous injection.

One of the animals was injected 24 hours after the bile fistula operation with 1.45 mg desoxycholic acid. The excretion in the bile was measured and is shown in fig. 4. The activity appeared much faster than after the intraperitoneal administration. Each of the ten minutes fractions was dried and 0.2—0.7 mg of the dry substance was subjected to ascending paper chromatography

with isoprophylether : heptane 75 : 25 as moving phase. Only one spot of activity corresponding to glycodesoxycholic acid appeared. Small amounts of activity at the starting point ("tauro-spots") could be detected on some of the chromatograms. No free acid and no glycocholic acid could be found. All the fractions were combined and hydrolyzed and the free acids separated with reversed phase chromatography using 55 per cent methanol as moving phase and chloroform : heptane 9 : 1 as stationary phase. Activity appeared only at the place for desoxycholic acid and recrystallization with unlabelled desoxycholic acid gave the following results:

Weight of crystals	Crystallized from	c. p. m. per mg
1 98 mg	Acetic acid/water	732
2 66 »	»	773
3 44 »	Ethyl acetate/petrol ether	748
4 33 »	Ethanol/water	765
5 21 »	»	756

The other rabbit was injected with 5 mg of the labelled desoxycholic acid 3 hours after the operation. 88 per cent of the activity was excreted in the first six hours and after 18 hours practically all activity had come out in the bile. Chromatography of the bile acids in system C (NORMAN 1953) gave exactly the same distribution of the activity as shown in fig. 2. No free acids were present and 6 recrystallizations of the "glycodesoxycholic acid band" with unlabelled glycodesoxycholic acid did not change the specific activity.

Discussion.

It is clear from the results that desoxycholic acid does not form cholic acid at the same rate in the rabbit as in the rat. In our experiments, however, the desoxycholic acid has only gone through the liver once and if a very small undetected conversion to cholic acid takes place this would give rise to more cholic acid after each passage through the liver in the entero-hepatic circulation. In that case, however, almost no cholic acid should be present in the bile when the preformed bile acids have been drained out through the bile fistula for the 24 hours preceding the experiment. The glycocholic acid titration peak in the chromatogram is, however, still present.

The results on the conjugation give additional evidence for the absence of free bile acids in the normal bile (AHRENS and CRAIG 1952, NORMAN 1954).

Summary.

The bile acids in rabbit fistula bile have been studied with paper chromatography and reversed phase partition chromatography.

The fate of administered desoxycholic acid-24-^{14}C has been studied. The major part of the acid is excreted in the bile as glycodesoxycholic acid. No transformation to cholic acid has been found to take place.

The technical assistance of Mrs. Ann-Mari Andersson is gratefully acknowledged.

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